Biomonitoring Of Surface Waters Using Duckweed (*Lemna Minor* L.)

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Abstract

Water pollution by toxic micropollutants, which is predominantly the consequence of human activities (industry, agriculture and urbanisation) is one of the most critical problems concerning drinking water resources and environmental protection of water bodies. Usage of plant test species has proven essential in investigation, detection and quantification of toxic activity in the natural environment. Toxic effects were investigated in several surface waters (Sava River basin, Croatia) collected monthly over a 3 month-monitoring period. Duckweed (Lemna minor L.) is often used as a plant model because it is a widely spread monocot which multiplies rapidly. Heavy metals were determinated by atomic absorption spectrometry, while nutrients determination was conducted using spectrometry and ionic chromatography. The fitotoxic indicators (relative frond number, relative fresh weight, chlorophyll and carotenoid contents) were monitored after seven days of exposure. All samples of tested waters caused growth inhibition and decrease of chlorophyll and carotenoid contents. The biological effects of water samples appeared related to the physicochemical characteristics. Therefore, bioassays should be included, along with conventional chemical analysis, in water quality monitoring programs. The results also suggest that duckweed should be used in the biomonitoring of water quality because of its simplicity, sensitivity and cost-effectiveness.

Keywords: aquatic plant, growth, chlorophyll, toxicity

Introduction

Water of suitable quality and quantity is essential to all life forms. Increasing population and industrial activity have focussed public attention on water quality and the need to monitor and protect the resource. This has prompted changes in legislation to control water pollution, and environmental agencies have been created to manage the resource. Water quality monitoring has traditionally been carried out by provincial and federal government agencies, municipalities, industry, and researchers at academic institutions. One of the main reasons for water quality monitoring, is to assess human influence on aquatic ecosystems. Human influence on water quality is often thought of in simple terms, such as discharging wastes from sewage collection systems or industrial outfalls. Without doubt, these "point sources" can have major impacts on receiving waters. More difficult to deal with are problems emanating from diffuse, "non-point" sources. Examples of nonpoint sources include acid precipitation being generated often thousands of kilometres away, siltation of streams caused by logging and agriculture, the input of nutrients from agricultural fertilization of fields, and urban runoff. The changes in water quality which are caused by these activities are often cumulative in effect and difficult to remedy because of the widely scattered sources.

Surface waters, as complex environmental mixtures, contain enormous number of potentially polluting substances. Therefore, physicochemical analysis alone is insufficient to provide the information about water quality and 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. Plant assays are highly sensitive to many environmental pollutants, including heavy metals and have been used for monitoring the potential synergistic effects of mixtures of pollutants (Wang and Freemark 1995). Duckweed is an aquatic, floating plant and is relevent to many aquatic environments, including lakes, streams, effluents and sediment. Duckweed plants are widely distributed in the world from the tropical to the temperate zones, from fresh-water to brackish estuaries, and throughout a wide range of trophic conditions. Duckweeds (Lemnaceae) possess physiological properties (small size, high multiplication rates, and vegetative propagation), which make them an ideal test system. Moreover, duckweed can be used in a wide range of pH-values (pH 3.5-10).

Duckweed (*Lemna minor* L.) is used in water quality studies to monitor heavy metals and other aquatic pollutants, because it may selectively accumulate certain chemicals and may serve as biological monitors (Axtell et al. 2003). In the present study, the standardised protocol ISO 20079 (2006) 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.
Many endpoints have been used to express duckweed test results. These endpoints are generally based on the population of duckweed plants such as frond number, plant number, root number, dry or fresh biomass, root length and frond diameter. Under stress conditions, duckweed plants can also exhibit many symptoms like chlorosis (loss of pigment), necrosis (localized dead tissue), colony breakup or root destruction. Biomarkers such as chlorophylls and carotenoids contents and enzyme activities, like peroxidase are commonly used as endpoints for toxicity tests.
The objective of the present study was to evaluate the sensitivity of the selected endpoints for screening and biomonitoring complex effluent samples as well as to compare the bioassays with conventional chemical analysis.

Methodology applied

The chosen sampling sites (Fig. 1) are from the Sava River basin and are part of a systematic water quality monitoring program performed on a monthly basis. The monitoring stations were as follows: Sava Jesenice (SJ) – the Sava River downstream of the Slovenian town, Jesenice, that produces municipal wastewater; Sava Županja (SZ) – the Sava River downstream from a sugar factory near the Serbian border; and Sutla (a tributary to Sava River) Prišlin (SP) – downstream from a glass industry. Each surface sample was collected monthly over a three-month period (from April to June 2008).

Physicochemical parameter analysis

Conductivity (µS/cm) and pH were measured *in situ*. The samples were maintained at 4 °C until the bioassays were carried out. Chemical analyses included chemical oxygen demand (COD, mg O_2/L), biological oxygen demand (BOD, mg O_2/L), suspended solids (SS, mg/L), nitrate (mg N/L), nitrite (mg N /L), ammonium (mg N/L), total nitrogen (mg N/L) and total phosphorus (mg P/L). The analyses were carried out 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). These routinely measured water quality indicators are presented as the mean of three individual values measured monthly over a three-month period (Table 1). The analysis of heavy metals was performed by atomic absorption spectrophotometry (Perkin Elmer AA 600) after microwave wet digestion (Anton Paar Multiwave 3000) of the dried and powdered material in 10 ml of supra-pure concentrated HNO_3 at 230°C.

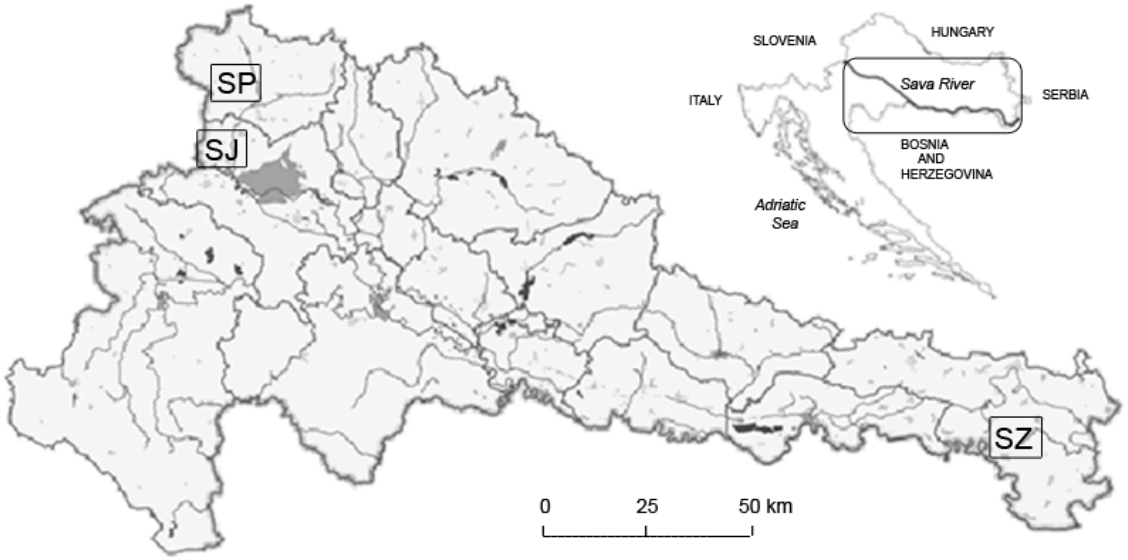

Figure 1. Sava river basin and monitoring stations

Plant material and experimental design

Lemna minor L. 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 Erlenmayer flasks containing either tested surface waters or dH_2O (control). All water samples were supplemented

with Steinberg macro- and microelements (1946) and prior to that filtered using cellulose nitrate membranes (pore size 0.45 µm). 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.

Growth parameters

Duckweed growth was determined measuring frond number (FN) and fresh weight (FW, biomass) according to the ISO 20079 test protocol. 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. Relative growth rate (RGR) was calculated from the following equation with the measured parameter *x* (FN, FW) and the start of the test (t0) for each replicate separately: RGR = (ln x_{t1} – ln x_{t0})/ t1-t0.

Chlorophylls and carotenoid contents

Chlorophyll *a* (chl *a*), *b* (chl *b*) and carotenoid contents were measured according to the method described by Arnon (1949). In short, 30 mg fresh samples were homogenized with 80% (w/v) cold acetone, centrifuged at 5 000 x *g* 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).

Statistical analysis

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

Results obtained

The levels of the physicochemical parameters are presented in Table 1. A pH range from 6.5 to 8.5 pH units is acceptable in drinking water. The pH levels of the surface water samples collected from three stations were alkaline (between 7.95 and 8.4) which indicates the presence of carbonates, hydroxides and bicarbonates. The base flow of a waterway acquires mineral constituents in the form of dissolved salts in solution, such as sodium, chloride, magnesium, sulphate, etc. In periods of high surface runoff, overland flow contributes dissolved materials to waters. In addition, significant contributions to the suspended solids load are anthropogenic in the form of municipal and industrial effluents, agricultural runoff, and aerosol fallout. High concentrations of suspended solids limit the suitability of water as a drinking source. The highest values of SS were measured in the sample of the Sava River downstream from a sugar factory (SZ). However, in general, the values of SS in water samples collected from monitoring stations over a 3-month period were relatively low. Salts, minerals, and even dissolved gases contribute uniformly to the conductivity of a solution. Electrical conductivity of water is a simple and useful indicator of the amount of dissolved materials in a solution. The slightly higher conductivity values, in comparison to other samples, were detected in the Sutla River sampled downstream from a glass industry (SP). The highest concentrations of the other chemical indicators (COD, BOD, total N, total P and especially ammonium) were also detected in the water samples SP.

Table 1. Physicochemical analysis of surface water samples collected over a 3-month period.

	1st month			2nd month			3rd month		
Parameter	**SJ**	**SZ**	**SP**	**SJ**	**SZ**	**SP**	**SJ**	**SZ**	**SP**
pH	8.07	7.95	8.03	8.15	8.1	8.27	8.15	7.97	8.4
Conductivity (µS / cm)	541	428	535	426	403	567	558	404	536
COD (mg / L)	2	2.1	2.5	2.1	2.2	2.8	2.8	3.2	4.1
BOD (mg / L)	0.9	1.5	1.6	1	1.9	2.4	1.4	2.3	3.2
SS (mg / L)	2.2	6.4	1	1.4	6.4	2.6	4	6.4	2.2
Nitrate (mg / L)	1.09	1.08	1.1	1.34	1.02	0.82	1.09	1.46	1.32
Nitrite (mg / L)	0.01	0.02	0.03	0.01	0.02	0.01	0.01	0.025	0.02
Ammonium (mg / L)	0.04	0.1	0.3	0.06	0.08	0.44	0.1	0.08	0.21
Total N (mg / L)	1.35	1.27	1.62	1.67	1.35	1.78	1.3	2.16	2.17
Total P (mg / L)	0.09	0.11	0.15	0.07	0.09	0.12	0.17	0.09	0.38

The analysis of heavy metals is shown in Table 2. The concentrations of Ni and Cr over a 3-month monitoring period were, on average, the highest in the Sava River downstream from a sugar factory (SZ) while those of Pb and Hg in the Sutla River. When compared to other water samples, the

concentrations of all measured heavy metals were the highest in water sample SP collected at the end of monitoring period.

Table 2. Concentration of certain heavy metals in the samples of surface waters collected over a 3-month period.

µg/L	1st month			2nd month			3rd month		
	SJ	SZ	SP	SJ	SZ	SP	SJ	SZ	SP
Zn	2.19	2.7	2.92	2.77	5.69	2.19	1.06	3.07	7.05
Cu	0.8	0.77	0.95	0.83	1.35	1.14	0.63	0.76	2.99
Ni	1.28	4.37	3.5	3.78	3.44	1.12	4.69	7.27	7.67
Pb	0.05	0.03	0.2	0.04	0.07	0.23	0.05	0.07	0.23
Hg	0.01	0.017	0.01	0.015	0.016	0.022	0.01	0.01	0.02
Cd	0.009	0.009	0.07	0.02	0.04	0.04	0.008	0.005	0.27
Cr	0.58	2.24	0.92	1.23	1.2	0.39	2.08	2.35	3.23

All tested water samples (SJ, SZ and SP) caused the inhibition of relative growth rate based on FN which averaged 32% for SJ, 35% for SZ and 25% for SP over a 3-month monitoring period (Fig. 2). The same trend of growth inhibition, but to a much lesser extent, was noticed with biomass. Significant decrease in RGR based on biomass was recorded in plants grown on almost all water samples except SJ and SP collected after first month and SP collected after second month (Fig. 2).

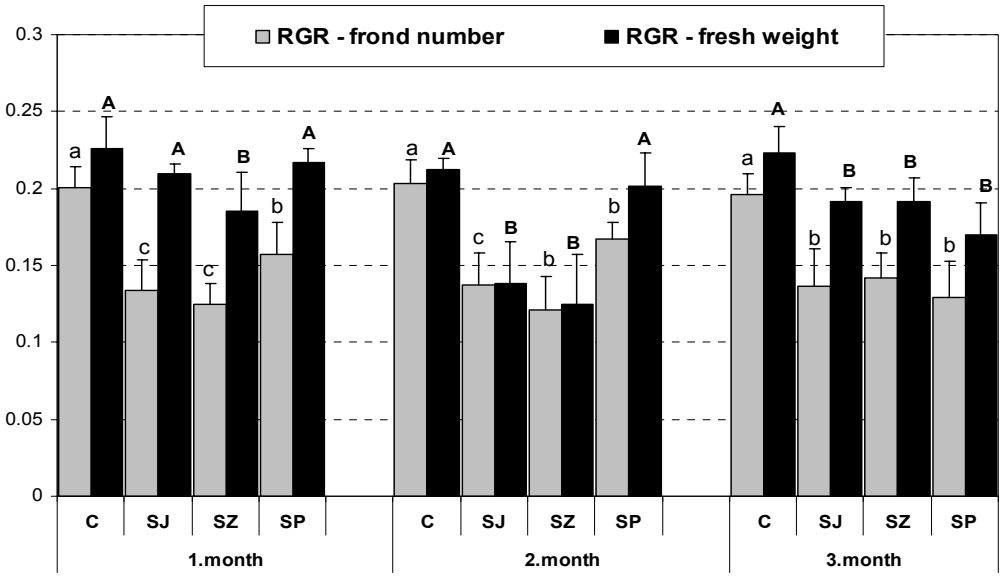

Figure 2. Relative growth rate based on frond number and fresh weight in control duckweed (C) and duckweed grown 7d on water samples (SJ, SZ, SP) collected over a 3-month period. Bars with different letters are significantly different at $p < 0.05$.

In the literature cited, frond number is considered to be the least reliable in comparison with other growth endpoints observed in (final biomass, frond area and dry weight) 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). However, in the present study frond number proved to be more sensitive parameter than 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 (7d) in landfill leachate.

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. Duckweed leaves started to show signs of chlorosis (pigment loss) following 7d exposure to surface water samples. Accordingly, there was a marked decrease in chlorophyll and carotenoid contents, especially that of chl *a* (Fig. 3A). The carotenoids serve as

antioxidant molecules which 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 plants following exposure to Cu, Pb and Ni (Axtell et al. 2003).

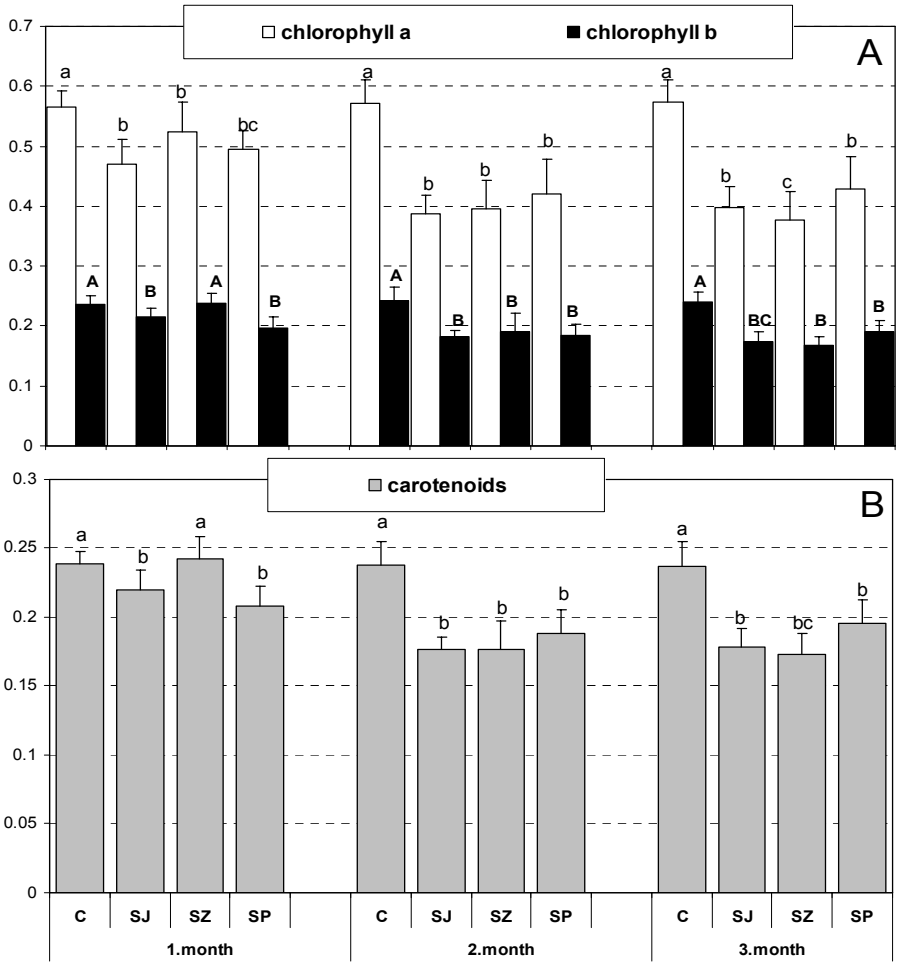

Figure 3. (A) Chlorophyll a and b, **(B)** and carotenoids contents (mg / g FW) in control duckweed (C) and duckweed grown 7d on water samples (SJ, SZ, SP) collected over a 3-month period. Bars with different letters are significantly different at $p < 0.05$.

Growth rates based on frond number correlated closely with the chlorophyll *a* content of duckweed exposed to monitored surface waters. Chl *b* degraded at a much slower rate than chl *a* which suggests greater damage of pollutants present in water samples on chl *a*.
The results show the suitability of *Lemna minor* L. for surface water quality assessment as selected endpoints showed consistency among each other and with respect to different water samples which were collected monthly over a 3-month period. The possible reason for such consistency among observed endpoints 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 replicate treated individuals. Moreover, water and substances to be tested are taken up directly through the leafy fronds (Naumann et al. 2007).

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 leads to a large standard deviation in results.

Due to their simplicity, sensitivity and cost-effectiveness toxicity tests with *Lemna minor* should be used in the biomonitoring of surface water quality. The results obtained also show the usefulness of combining physicochemical analysis with bioassays as such approach ensures better understanding of the toxicity of chemical pollutants and their influence on health.

Acknowledgements

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