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The use of computer image analysis in a *Lemna minor* L. bioassay

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Abstract Our study presents a low-cost method (no expensive hardware platforms required) of quantified biomonitoring based on computer image analysis. The negative influence of toxins on surface waters was analysed. The method was verified on widespread freshwater macrophyte *Lemna minor* to test populations treated with non-ionic detergents. We showed that the proposed automated bioassay has a broad applicability in assessing the negative impacts of aquatic toxicants. This approach enabled fast and precise evaluation of the morphometric parameters of the duckweed test population. We observed that growth rate of *L. minor* reacts to non-ionic detergents, which is reflected by the change in the surface area. The decrease in the growth of *L. minor* was revealed at high doses of detergents. This test proved to be highly useful, because it is well repeatable and fast in its implementation. Unlike classical bioassays, the proposed test allows the elimination of measurement errors, resulting from observers' subjectivity.

Keywords *Lemna minor* bioassay · Computer image analysis · Water pollution assessment · Bioindicators

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Introduction

Since the 1980s, biological methods of environmental monitoring have been developed intensively (Wolna-Maruwka et al., 2012; Amini & Kraatz, 2014; Debén et al., 2015; Fennessy et al., 2015; Harvey et al., 2015). Toxicity assessment of samples collected from the environment requires the selection of appropriate biological indicators (Kuczyńska et al., 2003; Uherek et al., 2014; Ibáñez et al., 2015), i.e. organisms particularly sensitive to the effects of specific pollutants, and the toxic effects must be indicated as clearly defined symptoms (Walker et al., 2002, 2013; Kumar et al., 2015). The majority of bioassays are based on a visual observation approach, where the observer evaluates symptoms of the toxic impact of pollution on bioindicators (Wang &

Freemark, 1995). A wide range of various bioindicators or biosensors (enzymes, physiological responses, etc.) have been applied to this kind of monitoring (Dobrowolski et al., 2012; Narwaria & Saksena, 2012; Busquet et al., 2014; Wang et al., 2014). On the other hand, still a relatively limited range of biomonitoring methods are based on an automatic approach, where the subjectivity of observations is usually significantly reduced. This approach to observations often involves the use of computer image analysis methods in bioassays (Mazur & Lewicki, 2008; Cerbin et al., 2012; Wells et al., 2012). This kind of method allows objective evaluation of the toxicity of a tested sample.

The development of image analysis procedures enables semi-automatic or fully automatic measurements. The computer records the changes and defines their nature, both qualitatively and quantitatively. In this context, it is important to find a technique for extracting information encoded in a graphical form, generally based on the characteristics of predefined features. To extract information exhibited by living organisms recorded in the form of digital images, various advanced analytical tools are used (Nowakowski et al., 2009, 2011; Sozzani et al., 2014) including advanced forms of digital simulators of brain function in the form of artificial neuron networks (Solarz et al., 2011).

The classical biotests utilising duckweed are based on dry plant biomass comparisons between the control and experimental samples. These are invasive methods and do not allow the continuation of the experiments, since the plants are harvested for biomass measurements. Another method is based on manual counting of the number of thalli ("leaves"), regardless of their size. A more reliable and more accurate method of defining the growth of biomass is comparison of the area of thalli in the control and experimental samples. This is a non-invasive method allowing the continuation of the experiment. Computer image analysis methods provide objective support for data collection (Rahaman et al., 2015; Agathokleous et al., 2016).

Our study aimed to test a low-cost approach to objective and quantified biomonitoring based on computer image analysis, reflecting the negative influence of toxins on surface waters, which can be carried out by a researcher without the need to purchase expensive hardware platforms. Our objective is to propose basic solutions for image analysis (standard digital camera) and framework software. The method was verified on test populations of the widespread freshwater macrophyte *Lemna minor* L., treated within a toxicity bioassay of non-ionic detergents. Our hypothesis was that the morphometric reaction of *L. minor* to non-ionic detergents could be detected with the computer image analysis approach, using a regular digital camera and Aphelion software.

Materials and methods

Lemna minor axenic culture preparation

Duckweed cultivation (stock culture) was based on Swedish standard (SIS) *Lemna* growth medium, prepared according to the standard procedure (OECD guideline for testing of chemicals, 2006, *Lemna* sp. growth inhibition test, No 221, p. 22). This protocol was followed to create an axenic culture of *Lemna* groups for experiment. The aquarium stock of *L. minor* axenic culture was maintained in a sterile 30 l glass tank at the Laboratory of Environmental Biotechnology and Ecology, AGH University of Science and Technology in Krakow, between October 2012 and October 2013. Calibration was completed in June 2016. Each aquarium containing the sterile medium was continuously aerated. The physicochemical parameters—pH, temperature and photoperiod—were controlled. All duckweed plants selected for the test had a high growth rate potential, were healthy, and had no visible symptoms of chlorosis on the leaves.

Method calibration

Calibration of the proposed method was carried out experimentally to test the correlations between the leaf surface area of the *L. minor* test populations and their weight, in an experiment consisting of 36 measuring series (with three replications). The 36 measuring series in three replications were differentiated according to the number of duckweed individuals. The image analysis procedure was carried out by measuring the frond surface area of each population, and the fresh biomass weight was estimated for them. The results were presented graphically, and the regression curve was also plotted. The correlation coefficient was

calculated, and statistical analysis was performed for the results.

Experiment with detergent

The experiment was based on the application of non-ionic detergents belonging to the Brij series to common duckweed (*L. minor*). Detergents are the source of many environmental problems. They deteriorate the oxygen balance in waters, and consequently disrupt self-purification processes (Mazur et al., 2013). We used Brij series detergent due to its long period of biodegradation, which has a prolonged impact on aquatic biota.

The equipment used for the toxicological tests consisted of a phytotron chamber, where beakers with the control and experimental groups were placed against a black background. The light intensity ranged from 1458 to 1499 lx; thus, it was very homogeneous on the surface (Fig. 1). The reflexes from the walls of the beakers were not eliminated; however, they did not have any influence on the changes in the growth of *L. minor*. The figure presents a digital network with the light values in lx, measured with a Digital Lux Meter GM1020.

The images were taken using a Nikon Coolpix 995 digital camera. The camera was mounted on a stand to maintain a consistent and equal distance from the plants during each experiment. The tests were conducted in 300 ml glass beakers, with 250 ml of liquid in each. Ten individuals were transferred to each beaker containing different concentrations of the test detergents, and the containers were covered with Petri dishes to prevent excessive evaporation. Six concentrations for each toxicant and the control group were used for determination of the negative toxic effect of the bioassay. Each experiment was conducted in four replications.

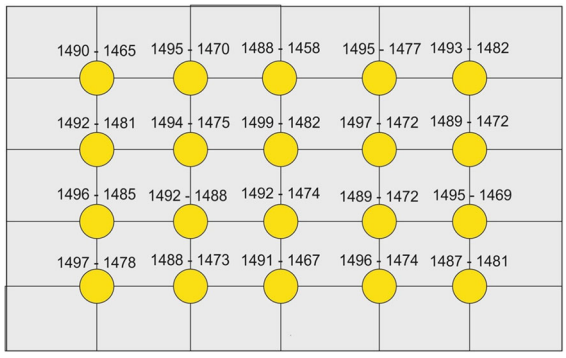

Fig. 1 The lightening conditions of the phytotron chamber with experimental groups in beakers, ranges of light intensity (in lx)

Stock solutions of the toxicants were prepared in a large volume at a standardised concentration, and were diluted to lower concentrations for the chronic toxicity bioassay. The solutions of detergents Brij 58 and Brij 35 were made in geometric progression, with respective ratios of $q = 1.5$ for Brij 58 and $q = 1.25$ for Brij 35.

For Brij 58, the standardised concentration was 1991 mg dm^{-3}, and the solutions were diluted in a geometric progression with ratio $q = 1.5$ up to a concentration of 262 mg dm^{-3}. Tests were performed on one control group and seven experimental groups.

For Brij 35, the standardised concentration was 800 mg dm^{-3}, and this was diluted in a geometric progression with ratio $q = 1.25$ up to a concentration of 210 mg dm^{-3}. There were eight experimental groups and one control group. The solution coefficients were selected based on pilot tests, which enabled preliminary estimation of toxic effects for the studied detergents.

Test populations of ten individuals of *L. minor* were placed in each solution sample (one population per tray). Photographs of the *L. minor* plants were taken for each test group (with fixed camera settings and distances). Each image was analysed using Aphelion 3.02, and results were obtained as the average surface area of the entire test population in pixels. A macro program for automatic image analysis of a series of images was used (Fig. 2).

The algorithm begins with loading the image to be analysed. Then, a median filter was applied to reduce noise. In the next step, the colour image was converted to a monochromatic one. Breakdown of the image into RGB components was used. The blue component was used for further analysis, as it provides the highest contrast for the duckweed. In the next step, the Black Top Hat filter was used for better mining of the objects. This was followed by binarisation (corrected entropy threshold) and removal of small objects considered to be noise (erosion + image reconstruction). Next, the area with duckweed was defined (image acquisition). The tray with the duckweed was outlined in black to define parts of the plants. This enabled us to estimate the surface area for the elements inside the outline. In the final step, the objects identified by the program were outlined in

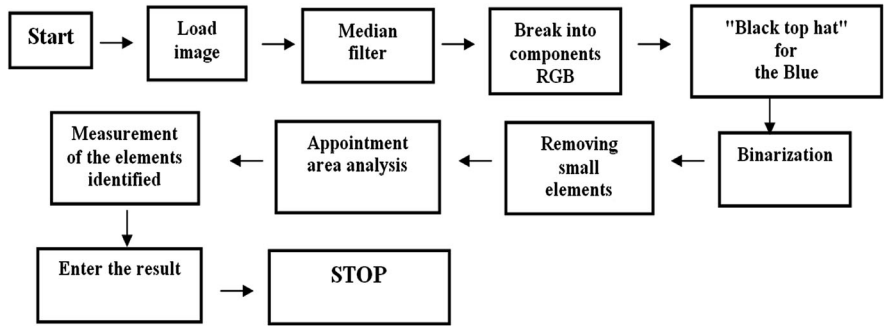

Fig. 2 Block diagram of the duckweed detection algorithm

order to verify their recognition by the user, and the results were entered (Fig. 2), (Mazur & Lewicki, 2008).

Exposure to toxicants in specific concentrations lasted 4 weeks, and five images were taken every 7 days in every test group. Glass covers were used to protect the trays with the duckweed against evaporation, while not restricting exposure to light. The experiment was carried out under strictly controlled conditions, maintaining fixed settings, while each group of *L. minor* was photographed: 12/12 photoperiod, temperature 24 ± 2 °C.

The results were analysed and compared within groups, between groups and with the control groups, using Statistica 10 statistical software.

Results

The calibration of the method showed a strong relationship between *L. minor* surface area and the plant biomass in each group (Fig. 3). The correlation coefficient was $R^2 = 0.996$, which permits the assumption that the *L. minor* frond surface area is directly proportional to the mass of the test group. The

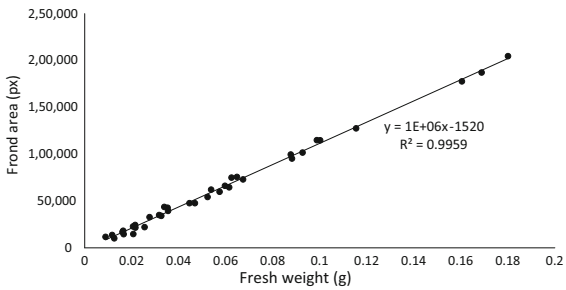

Fig. 3 Linear regression *curve plot* for frond area (px) and fresh weight (g) of each experimental group of *Lemna minor*

image analysis reflects the plant biomass very well. The results showed that, in testing the effect of pollutant toxicity on the duckweed population, both of these measures may be used interchangeably.

The principal results of the experiments on the reaction of *L. minor* to various detergent solutions (Brij 59 and Brij 38) showed that the different types of detergents resulted in different growth patterns at low doses, but high detergent applications always caused severe reduction in growth (Tables 1, 3).

The largest surface area of *L. minor* was recorded for the concentration of Brij 58 equal to 393 mg dm^{-3}. Increasing detergent concentration beyond this point caused a significant drop in *L. minor* surface area. Plant surface areas at the highest concentration were significantly lower than in the control (Table 1; Fig. 5).

Differences in *L. minor* surface area in various stages of the experiment with Brij 58 applications are presented graphically using a box whiskers plot (Fig. 4). The non-outlier range and quartile values of *L. minor* are presented. This shows that a significant reaction to detergent applications requires at least a four-week period (Fig. 5).

There were significant differences in the growth dynamics of *L. minor*, depending on different detergent applications (Fig. 6).

The normal distribution of the analysed data was confirmed, and the assumption of homogeneity of variance was supported by Levene's test ($F = 1.699$, $P = 0.171$). Therefore, we used parametric ANOVA with post hoc Tukey's HSD. ANOVA showed the significance of the interaction effect, and the Tukey's test indicated a significant difference between each pair (Table 2).

Even the smallest application of Brij 35 caused a significant drop in *L. minor* surface area (Table 3).

Table 1 Average value (±1 SD) of *Lemna minor* fronds area exposed to different concentration of Brij 58 (during 1 month observation)

Detergent concentration (mg l^{-1})	1 week		2 weeks		3 weeks		4 weeks	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	17,601.3	32	20,402.3	397	25,788.5	5223	33020.4	163
262	18,810	496	19,073	818	25,987	1090	39453.6	429
393	17,690.5	1237	18,375.2	2641	27,764.6	1569	45508.9	1235
590	17,570.67	382	16,778.08	606	24,262.33	1806	40377	1345
885	19,283.17	1444	20,486.42	2796	26,376.83	1802	36608.4	1379
1328	18,548.17	1064	21,860.33	2818	27,114.83	3576	33515.5	299
1991	18,447.42	1475	20,519.83	2444	24,201.92	1797	26864.1	168

Results of fronds area given in pixels number (px)

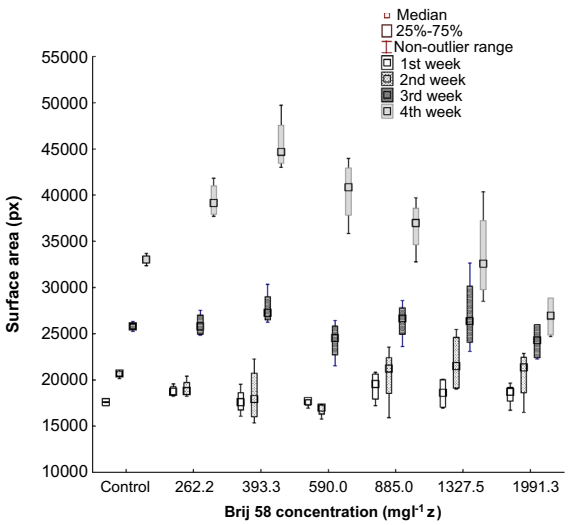

Fig. 4 Box whiskers plot: *Lemna minor* surface growth (in pixels) under different concentrations of the detergent during the four-week experiment

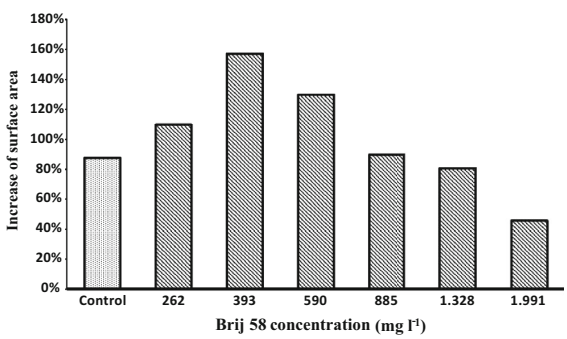

Fig. 5 The growth dynamics of *Lemna minor* exposed to Brij 58 solutions in each test group after 4 weeks of experiment

In analysis of the data from Brij 35 applications, the absence of normal distribution and heterogeneity of variance was detected by Levene's test ($F = 1.699$, $P = 0.171$). After log transformation, the variance was still heterogeneous, so a non-parametric test (Kruskal–Wallis) was used to define the nature of the differences between the test groups and the control (Table 4).

Discussion

The method of computer image analysis was used to study acute toxicity in a modified *L. minor* bioassay. The classical method is a widely used bioassay in the assessment of surface water quality and the potential impact of toxic agents—ISO 20079:2005: 'water quality—determination of the toxic effect of water constituents and waste water on duckweed (*L. minor*)—duckweed growth inhibition test.' We showed that the *L. minor* bioassay supported by the computer image analysis approach for morphometric measurements of plant surface area marks new standards for a more objective and quantified assessment of the negative influence of toxins on bioindicators. Automation greatly increases the speed and accuracy of the analysis, meaning that the test can be used on an industrial scale for quality assessment of surface waters in biomonitoring.

We have confirmed that *L. minor* reacts to non-ionic detergents by way of change in the plant surface area. Moreover, we found that high applications of detergents decrease the growth of *L. minor,* regardless

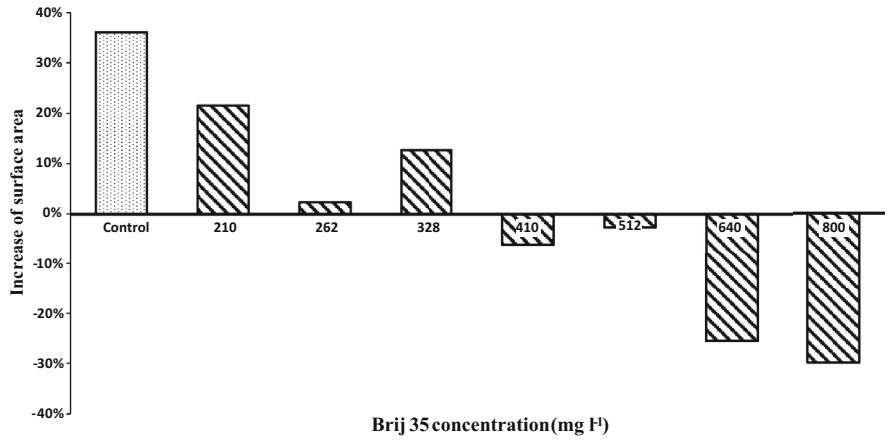

Fig. 6 The growth dynamics of *Lemna minor* exposed to Brij 35 solutions in each test group after 4 weeks of experiment

Table 2 Results of Tukey's HSD test for significance of differences between the test populations of *Lemna minor* exposed to different concentrations of Brij 58 solutions

Detergent concentration (mg l^{-1})	Control	262	393	590	885	1328	1991
Control		0.084	0.000	0.035	0.641	0.999	0.098
262	0.084		0.118	0.999	0.833	0.130	0.000
393	0.000	0.118		0.251	0.007	0.000	0.000
590	0.035	0.999	0.251		0.589	0.056	0.000
885	0.641	0.833	0.007	0.589		0.774	0.003
1328	0.999	0.130	0.000	0.056	0.774		0.068
1991	0.098	0.000	0.000	0.000	0.003	0.069	

$P = 0.012$

Table 3 Average value (±1 SD) of *Lemna minor* fronds area exposed to different concentration of Brij 35 (during 1 month observation)

Detergent concentration (mg l^{-1})	1 week		2 weeks		3 weeks		4 weeks	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	16,977	167	19,104	257	20,991	111	23,103	554
210	19,898	789	22,939	1096	24,074	1328	24,182	1786
262	19,421	897	19,897	1441	20,231	1533	19,876	1764
328	18,445	876	18,958	2443	20,718	3634	20,787	2818
410	18,349	1370	17,073	1210	18,495	2655	17,226	2863
512	19,781	887	18,269	552	19,967	4311	19,256	3713
640	16,444	2075	14,263	635	14,334	1178	12,249	2402
800	17,645	2355	17,642	2026	16,374	4363	12,383	2048

Results of fronds area given in pixels number (px)

of the type of substance (both Brij 35 and Brij 58). This effect can be used in biomonitoring of water pollution.

Some differences were found between *L. minor* development patterns on treatment with different tested detergents. Brij 35 detergent had a very aggressive impact on the duckweed populations. A distinct lethal effect was found at concentrations above 640 mg dm^{-3}, while concentrations of 412–509 mg dm^{-3} caused growth inhibition, and concentrations below 412 mg dm^{-3} were not toxic. Populations exposed to different concentrations of the detergent Brij 35 exhibited highly unstable growth (absence of homogeneity of variance).

Applications of Brij 58 were less toxic. Low applications of this detergent even showed a growth-

Table 4 Results of the Kruskal–Wallis test for significance of differences between the test populations of *Lemna minor* exposed to different concentrations of Brij 35 solutions

Detergent concentration (mg l^{-1})	Control	209.7	262.1	327.7	409.6	512.0	640.0	800.0
Control		1.000	1.000	1.000	0.545	1.000	0.013	0.008
209.7	1.000		1.000	1.000	0.602	1.000	0.015	0.010
262.1	1.000	1.000		1.000	1.000	1.000	1.000	1.000
327.7	1.000	1.000	1.000		1.000	1.000	0.807	0.602
409.6	0.545	0.602	1.000	1.000		1.000	1.000	1.000
512.0	1.000	1.000	1.000	1.000	1.000		1.000	1.000
640.0	0.013	0.015	1.000	0.807	1.000	1.000		1.000
800.0	0.008	0.010	1.000	0.602	1.000	1.000	1.000	

$P = 0.038$

stimulating effect. Concentrations of 393 and 590 mg dm^{-3} of Brij 58 produced statistically significant differences in biomass growth compared with the control. This was a stimulatory effect, which is confirmed by literature reports on the impact of detergents on eutrophication as a result of introducing biogenic elements to surface waters (Mazur et al., 2013). Applications of Brij 58 at a concentration of 1991 mg dm^{-3} reduced the growth rate of *L. minor*, exhibiting a toxic effect.

In our study, we have focused on *L. minor*, which is a widespread freshwater macrophyte that is advantageous for biomonitoring (Wang & Freemark, 1995). Brij applications have been widely used in toxicological experiments. Most toxicity tests for Brij detergents have been performed on higher organisms, but safety data sheets for chemical substances also require specification of acute toxicity for aquatic organisms (Noudeh et al., 2011; Williams et al., 2011; Beneito-Cambra et al., 2013; Li et al., 2013).

Our study showed the applicability of computer image analysis methods for toxicity bioassays with *L. minor* based on measuring the change in plant surface area. Moreover, the macro program analyses a series of images from a particular catalogue, and the data obtained are returned in a Microsoft Excel spreadsheet. This provides an extensive and comprehensive database that can be used in further statistical analyses of the investigated objects.

We have found that computer image analysis may provide important support in bioassays. Although the use of computer image analysis in water monitoring is currently gaining recognition, still few automated systems have been created and brought into use in biomonitoring (Streb et al., 2002; Akagi et al., 2014). The automated *L. minor* bioassay provides a new tool for assessment of the negative influence of aquatic toxicants. Present trends in aquatic ecotoxicology require toxicological assessment to be carried out with a wide range of bioassays (Garric et al., 1993; Zgorska et al., 2011; Forni et al., 2012; Testolin et al., 2012).

Conclusions

1. The growth rate of *L. minor* is affected by non-ionic detergents, as reflected by changes in the surface area.
2. A high concentration of detergents always reduced the growth of *L. minor*.
3. The morphometric reaction of *L. minor* to non-ionic detergents can be detected with the computer image analysis approach, using framework software and basic equipment.
4. The new *L. minor* bioassay with the application of computer image analysis for morphometric measurements of the plant surface area marks new standards for more objective and quantified assessment of the negative influence of toxins on bioindicators. The test can be used on an industrial scale for quality assessment of surface waters in biomonitoring.

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