

Growth response of *Lemna minor* L. to paracetamol

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Abstract: Surface water and groundwater is contaminated with pseudopersistent xenobiotics present in the environment. The risk associated with an increase in the consumption of drugs and their permanent occurrence especially in the aquatic environment falls on non-target organisms. The aim of this thesis was to assess the effect of increasing concentrations of paracetamol (0.1; 10; 100 µg/l) on the growth and selected physiological parameters of the model organism *Lemna minor* (L.). Duckweed, as an important model plant for ecotoxicological research, has been subjected to a semichronic exposure to paracetamol.

The presence of a stressor – paracetamol – after ten days of cultivation significantly influenced some growth and physiological parameters. It has been shown to significantly reduce the content of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, carotenoids), increase the value of non-photochemical quenching of chlorophyll fluorescence (NPQ), and lower the relative decline of chlorophyll fluorescence (Rfd). The results show that the increased load of paracetamol in the environment may negatively affect the growth of duckweed.

Key-Words: -chlorophyll fluorescence, duckweed, growth, *Lemna minor*, paracetamol, pharmaceuticals, photosynthetic pigments, phytotoxicity tests

Introduction

Plants have the ability to accumulate in their tissues toxic metals and organic pollutants, which can cause changes in growth and morphology. At present, drugs or pharmaceuticals include among the substances hazardous to living organisms [1, 2]. It is primarily about environmental contamination and wastewater sludge. It was found that in addition to acidification and eutrophication of soils and water may also indirectly contribute to the loss of plant and animal species. Paracetamol, often used as a representative of analgesics and antipyretics, is among the candidates for an analysis of the effect on a model organism *Lemna minor* (L.).

Pharmaceutically active substances are detected in surface water, groundwater and soil, wherein their concentration ranges from ng/l in µg/l [3], to small flows to 1 mg/l. These mobile elements of pollution enter the environment continuously [4] and their effect depends on the time during which the organism operates [5]. Studies also demonstrate that due to the high polarity and low volatility drugs bioaccumulate and are persistent in the environment [6].

Paracetamol is in 75% excreted as conjugates and in the remaining percentage the original substance persists [7]. The active paracetamol in these environments is easily transformed from conjugates

thanks to the activities of microbial metabolism. It is assumed that paracetamol pseudo-persistent in the environment [8]. The concentration of analgesics which include paracetamol in the aquatic environment varies between 0.0016 and 373 µg/l [9]. The average concentration of paracetamol was calculated in this study at 246 µg/l. Paracetamol and ibuprofen at the same time showed the highest mean values of the inflow to municipal wastewater treatment plants (38 and 37 µg/l respectively). Other researchers have found that the highest concentration reaches paracetamol in Europe compared with other continents (influent concentrations ranged from 59-220 ng/l, the concentration in rivers range from 12 to 777 ng/l) [10].

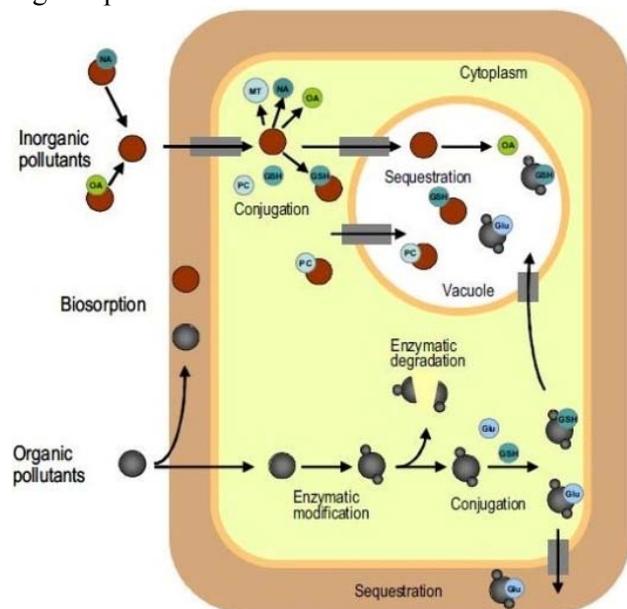
Removal of paracetamol in the WWTP is very effective in the order of 95% [11], yet the gate is paracetamol into the environment continuously. The concentration of paracetamol, which are normally determined in surface water can cause serious chronic effects on aquatic organisms [12]. Paracetamol has compared to ibuprofen a very low Kow value (0.40), is more accessible and therefore more toxic [13]. In particular, induction of oxidative stress may reduce the ability of effective degradation of paracetamol and other xenobiotics [14].

Hydrophyte communities - aquatic plants - form an important ecological element in the nutrient cycle.

They are part of the food chain of aquatic communities [15]. Interventions in the niches of aquatic plants may also have a negative impact on the surrounding biotes, including the quality of drinking water [16].

To protect water and aquatic ecosystems, which have far-reaching consequences for the entire biosphere, it is necessary to conduct toxicity testing of new and already used substances on aquatic plants. A preferred model organism for this purpose widely used due to its sensitivity and ability to accumulate is the higher aquatic vascular plant duckweed (*Lemna minor* L.) [17].

Fig. 1 Tolerance mechanism for inorganic and organic pollutants in *Lemna* cells.



Detoxification involves active sequestration in parts that can do least harm. The chelators involved are: glutathione (GSH); glucose (Glu); metallothioneins (MT); nicotinamines (NA); organic acids (OA) and polychelates. This is the reason why *Lemna* species accumulate high concentration of contaminants. Taken from [24].

Duckweed is used for the assessment of the ecotoxicity of target compounds in aquatic communities, mainly due to easy cultivation in the laboratory, small size, rapid vegetative propagation and reduced organization [18]. Sensitivity of common duckweed against often tested substances were compared with selected representatives of fauna and significantly clearer evidence of the presence of some toxic metals [19], pesticides [20] and pollutants, which are part of the wastewater [21] were obtained.

The conventional tests are carried out on the plant growth inhibition test by common duckweed growth curve. For ecotoxicological studies, this biomarker

provides basic data and information on the toxicity of substances or mixtures in aquatic ecosystem. Provides accurate data for determining the ecotoxicity of drugs or other substances [22]. The main advantage of this test lies in the plasticity of use and relatively low cost [23].

The aim of this paper is to analyse the effect of increasing concentration of paracetamol in a nutrient medium for vegetative growth of common duckweed. Paracetamol concentrations were chosen to correspond with the inlet into the continuous natural environment, and that the highest chosen concentration simulates a moderate degree of loading.

Material and Methods

Characterization of the model organism *Lemna minor* (L.) and experimental design

Cultivation of duckweed (*Lemna minor* L.) was carried out under controlled conditions (temperature $22 \pm 2^\circ\text{C}$, relative air humidity 60%, photoperiod 12/12, irradiance $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by white fluorescent tubes, Osram, Germany). The experiment was run in transparent 6-well plates (well volume of 12 ml, well surface area 9.62 cm^2 ; NUNC A/S, Denmark). Duckweed plants were placed into the plates (6 per well, i.e. 36 plants per plate) and cultivated in SM (10 ml per well) without (control) or with an addition of paracetamol ($0.1 - 10 - 100 \mu\text{g l}^{-1}$). Each treatment was represented by 6 plates. At the beginning of the experiment (day 0) and after 4 and 10 days of the cultivation the number of plants per well, fresh weight per plant, dry weight per plant, leaf area size per plant, the content of photosynthetic pigments per biomass unit and selected parameters of chlorophyll fluorescence of plants per well were assessed in each treatment.

Growth analysis

The number of duckweed plants was counted in each single well in all treatments and repetitions. Plants were then dried at 85°C for 6 h and their dry weight was determined and expressed as the mean per plant. Images captured during the chlorophyll fluorescence measurements (see Section Chlorophyll fluorescence measurements) were used for evaluation of size of leaf area. Values (as given in Fig.) represent the average leaf area per plant.

Chlorophyll fluorescence measurement

A set of chlorophyll fluorescence parameters (F_0 – basal chlorophyll fluorescence, F_v/F_m – potential yield of photochemical reactions in photosystem II, Φ_{II} – effective quantum yield of photosystem II, NPQ

– non-photochemical quenching, Rfd - ratio of chlorophyll fluorescence decrease) was determined from an analysis of slow kinetics supplemented with saturation pulses (recorded by a fluorescence imaging system HandyFluorCam, PSI, Czech Republic). This tool measured whole plates from each treatment in only one step. Measuring setup and calculation of selected parameters were adopted from Kummerová *et al.* (2007) [23]. The measurements were done at ambient temperature 21 ± 2 °C.

Analysis of photosynthetic pigments

For an analysis of photosynthetic pigments (chlorophyll *a* and *b*, carotenoids) approx. 0.03 g fresh duckweed plants (equal to approx. 36 plants) per treatment were extracted with 100% acetone and the pigment content was measured

spectrophotometrically (UV-VIS Spectrophotometer SPECORD 205, Jena, Germany) and calculated according to Lichtenthaler (1987) [25]. Pigment analysis was repeated three times for each treatment.

Statistics

For statistical evaluation of results, the software STATISTICA 10 (StatSoft Inc.®) was used. The results are means of at least three repetitions for all assessed parameters. The significance of the difference of the average values between the treatments was evaluated by one-way and multifactorial analysis of variance after verification of normality (Shapiro-Wilk test) and homogeneity (Cochran, Hartley, Bartlett test) of data variance (ANOVA, $P < 0.05$). The comparison of means was based on Scheffe test ($P \leq 0.05$).

Table 1 Results of selected parameters of chlorophyll fluorescence

Treatment ($\mu\text{g/l}$)	Day of cultivation	F_0	NPQ	Rfd
Control	0	70.56 ± 6.40	0.14 ± 0.01	0.97 ± 0.09
Control	10	69.16 ± 5.72^a	0.13 ± 0.01^a	0.91 ± 0.07^a
0.1	10	71.89 ± 6.59^a	0.26 ± 0.02^b	1.07 ± 0.09^b
10	10	79.37 ± 6.39^a	0.22 ± 0.02^c	0.52 ± 0.01^c
100	10	74.62 ± 6.43^a	0.22 ± 0.02^c	0.51 ± 0.04^c

Results and Discussion

In duckweed phytotoxicity tests usually the plant number and at least one more measurement variable, such as the total leaf area, fresh dry weight or the content of photosynthetic pigments are assessed (OECD, 2006; ČSN EN ISO, 20079, 2007) [26]. In this study after 10 days of culture, duckweed did not demonstrate growth inhibition for any of the concentrations of paracetamol (0.1 to 100 $\mu\text{g/l}$). Number of common duckweed plants during the experiment in the presence of paracetamol in comparison with control did not change significantly.

It is known that lower concentrations of xenobiotics can stimulate plants for a short period, as evidenced by the significant increase in the fresh weight of duckweed plants after 10 days of culture at a load of 10 $\mu\text{g/l}$ of paracetamol. However, in dry weight there were not detected significant differences in this nor in the other investigated variants. It can be assumed that the higher fresh weight related in this case only with higher water content in the tissues of duckweed. Also, a statistically significant increase in leaf area, which was shown at this load (10 $\mu\text{g/l}$ paracetamol) compared to the control variant, may

just be associated with increased water intake, which can lead to increased volume of cells.

In many studies, induction of oxidative stress by paracetamol with representatives of both vertebrates and invertebrates was observed [14]. Substance-induced oxidative stress can also contribute to inhibition of plant growth, as found in wheat plants or cucumber (*Cucumis sativus*). Zhang *et al.* [27] found that oxidative damage induced by cadmium was associated with decreased activity of antioxidant enzymes, which leads to increased production of oxygen free radicals which destabilize membranes. Paracetamol may, however, in the plant body behave differently. So far there are no relevant studies to confirm the results obtained in animal models.

Content of chlorophyll *a* and *b*, carotenoids

One of the negative consequences of the presence of xenobiotics is the reduction of content photosynthetic pigments [28]. Significant reduction of chlorophyll *a*, chlorophyll *b* and carotenoids when compared to control was observed after 10 days of duckweed culture, (Fig. 2, 3, 4) already at the lowest concentrations of paracetamol applied (0.1 $\mu\text{g/l}$).

Fig. 2 Content of chlorophyll *a* after 10 days cultivation.

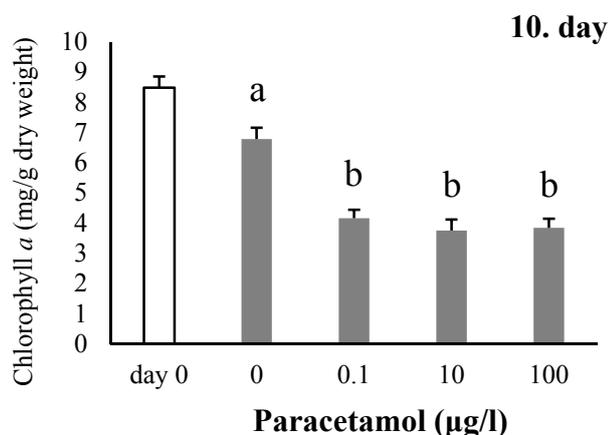
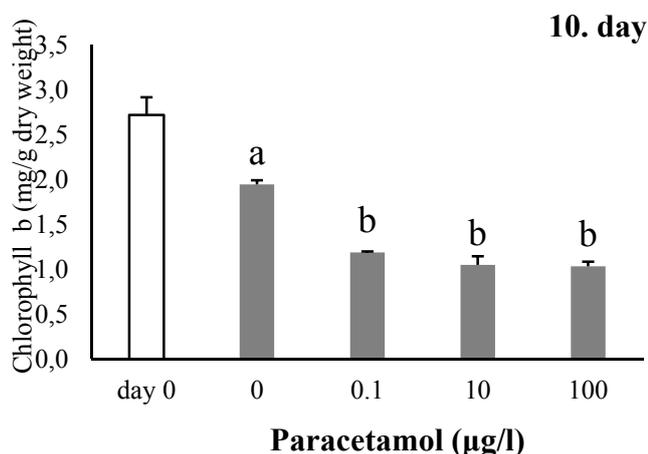


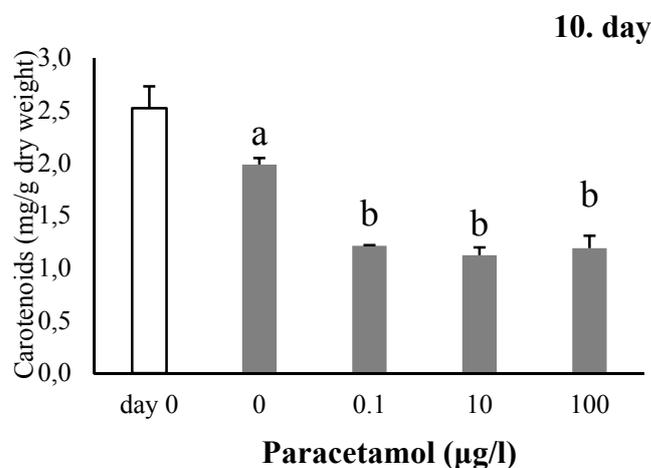
Fig. 3 Content of chlorophyll *b* after 10 days cultivation.



Azevedo *et al.* [29] reported that the reduction in chlorophyll content in leaves may occur both as a result of enzymatic degradation of these pigments (but none of the genes encoding the catabolic enzymes has yet been isolated), or by inhibiting their biosynthesis. This suggests that it may be one of the primary sites of xenobiotic metabolism toxicity in plants [30]. Carotenoids are according Hannoufa *et al.* [31] an important component of the photosynthetic systems. They are involved in both light absorption and protection of the chlorophyll molecule and plastid membranes from destruction by quenching of the triplet state of chlorophyll by removing oxygen in an excited oxygen-chlorophyll complex as antioxidants [32]. In our study, a significant reduction in the content of carotenoids of duckweed plants was observed, which thus cannot effectively protect the chlorophyll molecule. Reduced protective effect of carotenoids is also evident from the increase in the ratio of

chl*a*+*b*/carotenoids, which was recorded in comparison with the control.

Fig. 4 Content of carotenoids after 10 days cultivation.



Parameters of chlorophyll fluorescence

The value of minimal chlorophyll fluorescence (F_0) is due to stress conditions increases, while the effective quantum yield (Φ_{II}), expressing the rate of ongoing primary processes of photosynthesis, usually due to a stressor decreases. In our study, the value of basic fluorescence after 10 days of cultivation of duckweed in the presence of paracetamol did not change significantly (Table 1). No significant changes were observed either in terms of the maximum quantum yield of PSII (F_v/F_M) and effective quantum yield of PSII (Φ_{II}). We believe that the values of fluorescence were not sensitive enough to ensure that they are reflected in a reduced content of photosynthetic pigments.

Many authors have pointed out that the increase of non-photochemical quenching (NPQ) correlates with the presence of various stressors [33]. In our study, the value of this parameter compared to control increased significantly after 4 and 10 days of culture already at the lowest concentration (Table 1). These results demonstrate the possible stress effect of paracetamol.

Decrease of the relative chlorophyll fluorescence (R_{fd}) indicates higher levels of stress. This so-called vitality index is sensitive to the presence of a stressor more than the quantum yields. But it is not still clear, in what section of the photosynthetic apparatus stressor acts [34]. After ten days of culture, duckweed (Table 1) showed a significant increase in the value of the variant with 0.1 µg/l of paracetamol, which may be related to the mechanisms of acclimation to the stressor, and a significant reduction in the value of other loads (10 and 100 µg/l paracetamol).

The results presented in this paper contribute to the understanding of the impact of pharmaceuticals in plants, which are an important group of pollutants. This study evaluates the effect of paracetamol on growth and selected physiological parameters and their values however, reflect the changes occurring at the biochemical level. It can be assumed that the observed minor changes may be related to the low efficiency of selected concentrations of paracetamol on duckweed and the capacity of its defense mechanisms. It is clear, however, that due to the increasing environmental pollution by drugs and their unclear toxicological effects, special attention must be paid to this area.

Conclusion

Effect of increasing concentrations of paracetamol on the monitored physiological parameters of growth became more visible after ten days of cultivation. A significant increase in leaf area at all paracetamol concentrations (0.1 - 100 µg/l) was observed. Significant reduction of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, carotenoids) has been shown to apply already at the lowest concentration (0.1 µg/l paracetamol). The value of non-photochemical quenching of chlorophyll fluorescence (NPQ) for all paracetamol concentrations increased, and the relative decline of chlorophyll fluorescence (Rfd) with 10 µg/l and 100 µg/l of paracetamol decreased, which indicates the presence and impact of stressors.

The results obtained suggest that the increased paracetamol load in the environment can negatively affect the growth of common duckweed. In the future, it would be appropriate to focus on study of activities and content of selected enzymes involved in antioxidative mechanisms.

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