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Effects of Artificial Sweeteners on *Lemna minor*

Klára KOBETIČOVÁ[1], Klára Anna MOCOVÁ[1]*, Lucie MRHÁLKOVÁ[1] and Šárka PETROVÁ[2]*

[1]Department of Environmental Chemistry, Faculty of Technology and Chemistry, University of Chemistry and Technology Prague; [2]Institute of Experimental Botany AS CR, Prague, Czech Republic

**Corresponding authors: klara.kob@seznam.cz; klara.mocova@vscht.cz*

Abstract

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Artificial sweeteners are common micropollutants in the aquatic environment. They were detected both in surface waters and in groundwater. Human toxicity has also been studied quite intensively but their ecotoxicity has not been studied so far. To assess the impact of four artificial sweeteners (aspartame, sucralose, saccharine, and acesulfame K) and one natural sweetener (stevioside) on freshwater plants, a growth inhibition test was set up in the macrophyte duckweed (*Lemna minor*). Subsequently full dose-response curves were established by exposing *L. minor* plants to concentrations of each individual sweetener ranging from 6.25 mg/l up to 100 mg/l for 7 days. Three different endpoints were tested: frond number, frond area and total chlorophyll content. Tests were performed under sterile conditions. Sweeteners had various effects on *Lemna* plants. Saccharine, acesulfame K and stevioside did not cause any significant negative effects on any of the measured parameters. On the contrary, stevioside and saccharine caused slowly stimulative effects. Aspartame and sucralose inhibited growth parameters (frond number and frond area) but the chlorophyll content was not affected.

Keywords: acesulfame K; aquatic environment; aspartame; duckweed; saccharine; stevioside; sucralose; toxicity

Artificial sweeteners are added to various kinds of drinks and food, their average annual consumption in developed countries exceeded million tons (Smrčková & Bindzar 2014). They belong to so-called micropollutants, along with pharmaceuticals and cosmetics, because they have been measured in many natural matrices like in waste waters (Loos *et al*. 2009), surface waters (Gan *et al*. 2012), groundwater (Scheuer *et al*. 2014), ocean water (Sang *et al*. 2014) and drinking water (Ordóñez *et al*. 2012). Moreover, new sweeteners are still synthesized for their improved properties (Klescht *et al*. 2006). Use and replacement of natural sweeteners are advantageous primarily in economic terms (Zygler *et al*. 2011; Čopíková *et al*. 2013; Stolte *et al*. 2013). Artificial sweeteners can be divided into several groups. Most of them are chlorinated substances, in many cases cyclic structures (Smrčková & Bindzar 2014). Various studies deal with the influence of sweeteners on human health (Guidance for Industry and Other Stakeholders: Toxicological Principles for the Safety Assessment of Food Ingredients 2000). In recent years, research has focused on their ecotoxicity to aquatic or terrestrial organisms (Huggett & Stoddard 2011; Soh *et al*. 2011; Stolte *et al*. 2013; Kobetičová *et al*. 2016; Amy-Sagers *et al*. 2017). Sucralose ecotoxicity was not proved. It did not exhibit any adverse effects on the growth rate of *Lemna gibba* (7 days) at a concentration of 1000 mg/l (Soh *et al*. 2011). Moreover, Huggett and Stoddard (2011) found no significant reduction in survival or reproduction at concentrations up to

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1800 mg/l for *Daphnia magna* (21 days) or 93 mg/l for *Americamysis bahia* (28 days). Stolte *et al*. (2013) estimated LOEC values higher than 1000 mg/l for algae, daphnia, macrophytes, and activated sludge by six tested sweeteners. On the other hand, LOEC for aspartame and saccharine under a concentration of 100 mg/l active substance were described for Lemna minor in sub-chronic test and for *Enchytraeus crypticus* in reproduction test (Kobetičová *et al*. 2016).

Most of these studies have been focused on testing of chemically pure substances (Huggett & Stoddard 2011; Soh *et al*. 2011; Stolte *et al*. 2013), but artificial sweeteners get into the water mainly in the form of tablets or after use in the consumer industry. There are not many studies which took into account this issue. In a study the influence of aspartame and saccharine tablets on enchytraeid reproduction and growth rate of duckweed (*L. minor*) was found (Kobetičová *et al*. 2016). These diverse results therefore lead to different conclusions regarding potential ecotoxicity of artificial sweeteners and it is clear that further testing is necessary. Generally, the duckweed seems to be a very sensitive freshwater organism to aquatic contamination (Tóthová *et al*. 2007). This is a floating, emergent macrophyte, which belongs among the most commonly used organisms in ecotoxicology because of its rapid reproductive ability and easy cultivation (ISO 20079: 2005). It occurs abundantly in standing and slowly flowing waters, which provide shelter and food to some species of fish. It was also reported that it participated in the extraction of nitrate from water, thus reducing its amount in the ecosystem. It therefore plays quite a crucial role in freshwater systems (Štěpánková *et al*. 2010).

The aim of this study was to test the effect of five most frequently used sweeteners, aspartame, saccharine, sucralose, acesulfame K and stevioside on growth and biochemical parameters of duckweed in a laboratory ecotoxicity test.

MATERIAL AND METHODS

Test chemicals. Four artificial sweeteners which are commercially available and one natural sweetener were used as samples. Acesulfame K (99.9%) was obtained from Sigma-Aldrich (Czech Republic), Sucralose (5.4 mg/tablet) and Aspartame (9 mg/tablet) (F & N Ltd., Czech Republic), Stevioside (> 80%) (Institute of Public Health, Czech Republic) and Saccharine (16 mg/tablet) (Tišice Ltd., Czech Republic). Basic physicochemical properties are listed in Table 1.

Plant material and cultivation. *L. minor*, Steinberg culture originated from the Federal Environmental Agency (UBA, Germany). Sterile plant cultures were used for experiments. The plants were grown in Steinberg medium (ISO 20079:2005) and kept under constant conditions in a cultivation chamber at 20 ± 1°C and light regime of 16/8 h of light/dark.

Growth inhibition test. The experiments were performed according to ISO 20079 (2005). Healthy plants with total twelve fronds were transferred into 150-ml beakers filled with 100 ml of Steinberg nutrient solution. The experiment was carried out in quintuplicates (control) or triplicates (sweetener samples). The concentrations were 6.25, 12.5, 25, 50, and 100 mg/l for all tested sweeteners, with the exception of sucralose. This sweetener was tested twice and the tested concentrations were firstly the same as in the test with the other sweeteners and secondly at a range from 0.1 to 500 mg/l on the basis of results of the first sucralose test. The experiments were carried out over a period of 7 days under the same cultivation conditions (temperature 20 ± 1°C; light/dark regime was 16/8 h). Frond number and changes in their appearance were recorded photographically on the first, the fourth and the seventh day. The frond area was calculated by image analysis in NIS Elements 4.2. programme (2014). After seven days, total chlorophyll content was measured according to Wellburn (1994). The content of chlorophyll a and b and total

Table 1. Structural formulas, basic properties and determined concentrations of the sweeteners in Steinberg medium (Stolte *et al*. 2013) (*log – not analysed)

Sweetener	Chemical formula	M (g/mol)	log (K_{ow})	Water solubility (g/l)
Acesulfame	$C_4H_5NO_4S$	163.15	–1.33	270 (20°C)
Sucralose	$C_{12}H_{19}Cl_3O_8$	397.63	–1.00	283 (20°C)
Aspartame	$C_{14}H_{18}N_2O_5$	294.31	0.07	10 (25°C)
Saccharine	$C_7H_5NO_3S$	183.19	0.91	4 (20°C)
Stevioside	$C_{38}H_{60}O_{18}$	804.87	–*	1.25 (25°C)

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chlorophyll in *L. minor* was then calculated (Eq. 1–3). The chlorophyll content was calculated per leaf area unit (μg/cm^2).

$$\text{Chl } a = 15.65 \times A_{666} - 7.34 \times A_{653} \quad (1)$$

$$\text{Chl } b = 27.05 \times A_{653} - 11.21 \times A_{666} \quad (2)$$

where: A_{653} – absorbance of chlorophyll at a wavelength of 653 nm; A_{666} – absorbance of chlorophyll at a wavelength of 666 nm

$$\text{Chl}_{total} = \text{Chl}_{a+b} / A \quad (3)$$

where: Chl_{total} – total content of chlorophyll in the sample (μg); Chl_{a+b} – sum of chlorophyll *a* and chlorophyll *b*; A – frond area (cm^2)

The results were expressed as the percent inhibition compared to controls. For statistical evaluation of the results, the Graphpad software (2009) was used. The significance of the differences in the average values between the concentrations and controls (NOEC and LOEC values) was evaluated by the one-way analysis of variance (Dunnet Test, Anova, $P < 0.05$). The estimation of EC_{50} values was based on the method of non-linear regression ($P < 0.05$).

RESULTS AND DISCUSSION

This study on aquatic ecotoxicity of sweeteners builds on the previous work, in which the effect of aspartame and saccharine at a concentration of 100 mg/l on several species of aquatic organisms, i.e. freshwater alga (*Scenedesmus subspicatus*), duckweed (*Lemna minor*), mustard (*Sinapis alba*) and daphnia (*Daphnia magna*), was studied (Kobetičová *et al.* 2016). This work showed duckweed as the most sensitive test species. Therefore, it was chosen as a model organism in the present study.

Frond number. The results showed negative effects of sucralose and aspartame on the growth rate of fronds (Figure 1). The remaining sweeteners did not cause any significant negative effect on the frond number. In opposition, stevioside and partly saccharine had a stimulation effect on the growth of plants in comparison with controls (Figures 1 and 2). The ecotoxic effect was increased in this order: stevioside < saccharine < acesulfame K ≈ < aspartame < sucralose. The indices of toxicity (NOEC, LOEC and EC_{50} values) are listed in Table 2. Sucralose, saccharine and aspartame were tested in tablet form, containing

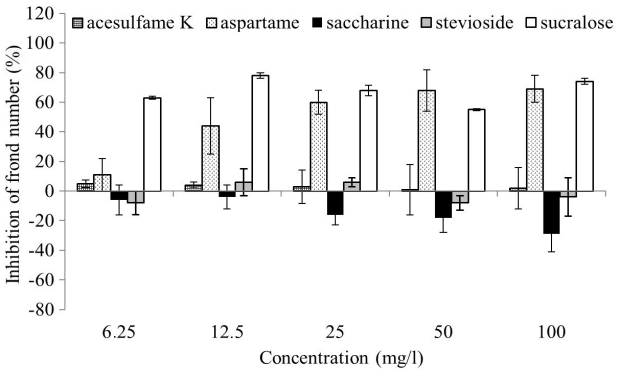

Figure 1. Inhibition (%) of the frond number (ind.) at tested concentrations in comparison with controls for tested sweeteners. The results are expressed as the percent inhibition compared to controls

also some additional substances besides the active sweetener. Saccharine tablets consist of sodium bicarbonate and tartaric acid, sucralose tablets of fructose, anti-caking agent: calcium phosphate and aspartame of sodium citrate, sodium bicarbonate, anti-caking agents: microcrystalline cellulose, magnesium stearate, etc. The concentrations of these additives are not indicated on the package, and for this reason it is not possible to discuss their potential environmental levels and effects. Some studies described the toxicity of some of these additives, e.g. sodium bicarbonate to fish (Farag *et al.* 2014; Harper *et al*. 2014), or the effect of sodium bicarbonate on toxicity of uranium (Soudek *et al*. 2011). Sucralose also has an effect on metal toxicity (Hu *et al*. 2016). For this reason, testing of tablets as well testing of pure sweeteners is relevant from an ecotoxicological point of view. It is possible that this can explain discrepancies between our results and other studies (Huggett & Stoddard 2011; Stolte *et al.* 2013).

Frond area. Growth area was increased along with an increase in the growth rate of duckweed fronds. Aspartame had negative effects on the frond area but saccharine, stevioside and acesulfame K did not (Figure 3A). This is in accordance with the study of Hu *et al*. (2016), who did not confirm the toxicity of acesulfame. Results from both tests with sucralose indicated an ecotoxicological effect from 0.1 to 500 mg/l (Table 2 and Figure 3A). The plants were affected in the increasing order from saccharine < stevioside < acesulfame K ≈ < aspartame < sucralose. In the case of sucralose, we tested also a concentration of 500 mg/l and this level caused 100% inhibition and fragmentation in this study (Figure 2E). This result was very different from the data in Stolte *et*

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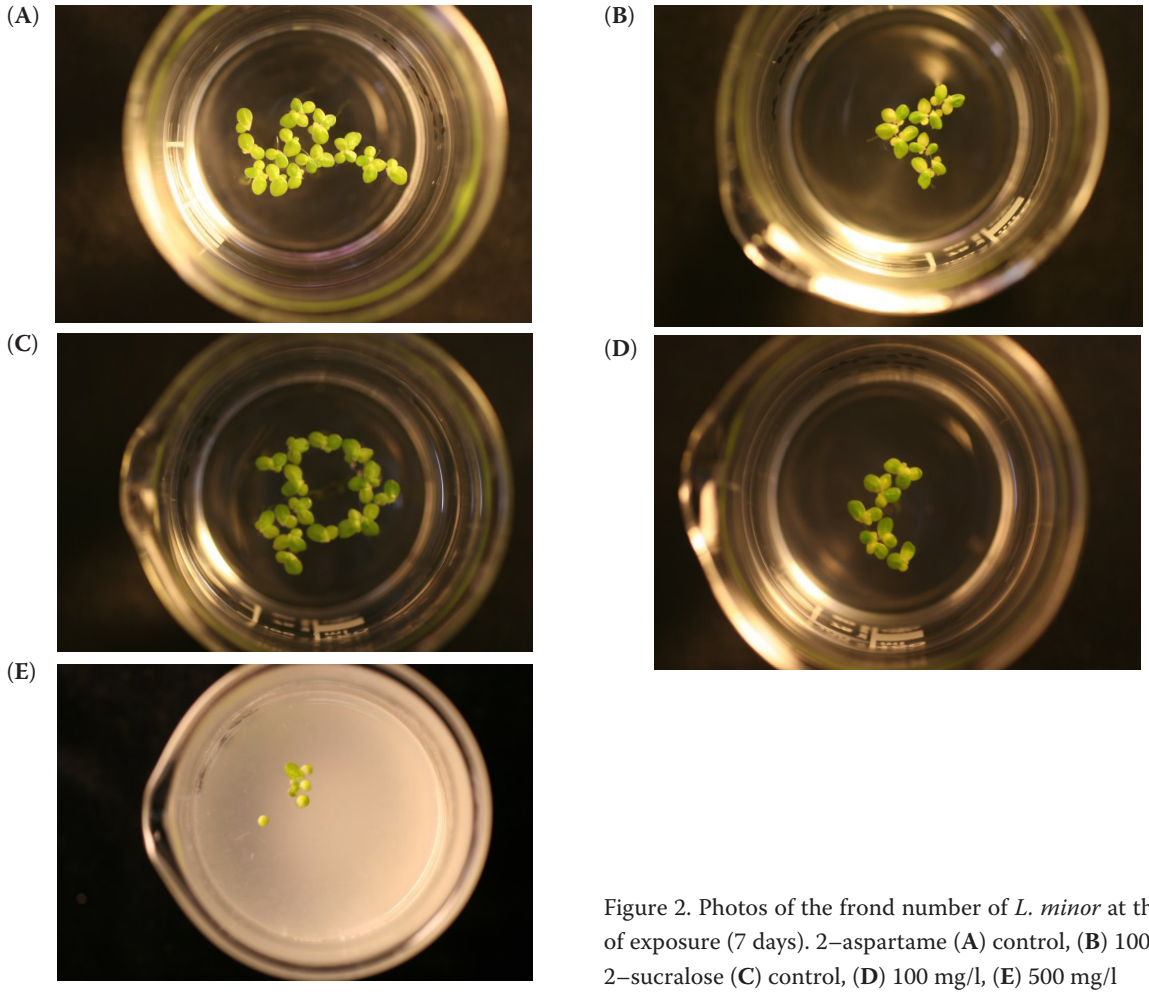

Figure 2. Photos of the frond number of *L. minor* at the end of exposure (7 days). 2–aspartame (**A**) control, (**B**) 100 mg/l; 2–sucralose (**C**) control, (**D**) 100 mg/l, (**E**) 500 mg/l

al. (2013), where LOEC was higher than 1000 mg/l and from the results of Amy-Sagers *et al.* (2017), where the authors used relevant environmental sucralose concentrations and their results indicated an increase of the frond area and use of sucralose as a source of carbon for plant metabolism.

Content of photosynthetic pigments. The results showed no significant ecotoxicological effects of any tested sweetener (Figure 3B). The ecotoxic indices are listed in Table 2. However, it is very interesting that in the present experiment there was a significant difference in chlorophyll content between control and aspartame solutions. It has been observed that the inhibitory effects increased with decreasing concentration in the solution (Figure 3B). It would be interesting to monitor the effects of aspartame

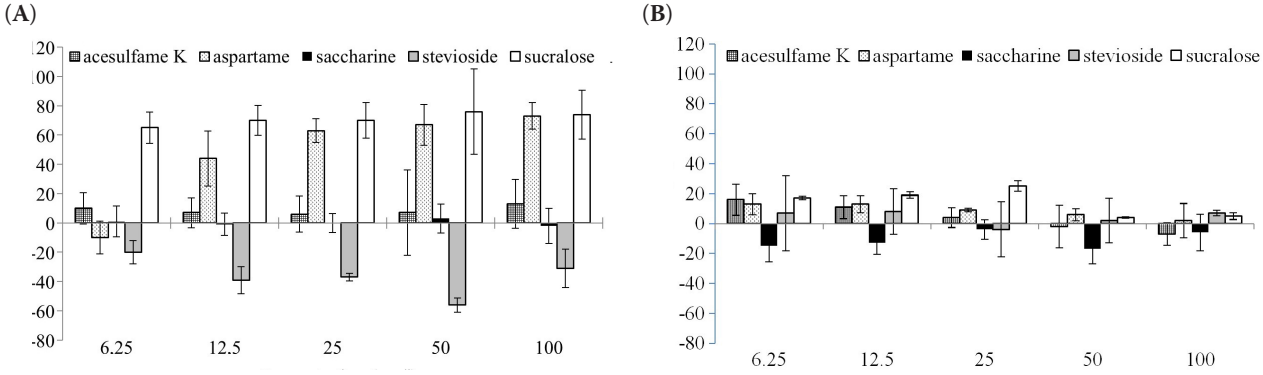

Figure 3. Inhibition (%) (**A**) of the frond area growth (cm^2) and (**B**) of chlorophyll content ($\mu g/cm^2$) at tested concentrations in comparison with controls for tested sweeteners. The results are expressed as the percent inhibition compared to controls

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Table 2. Toxicity indices for frond number, frond area and chlorophyll content of *L. minor*

Sweetener	Endpoint	NOEC	LOEC	EC_{50}
		(mg/l)		
Acesulfame	frond number	100	> 100	> 100
	frond area	100	> 100	> 100
	chlorophyll	100	> 100	> 100
Sucralose*	frond number	< 0.1	0.1	< 0.1
	frond area	5	10	< 500
	chlorophyll	> 500	500	> 500
Aspartame	frond number	6.25	12.5	11.62
	frond area	12.5	25	12.5–25
	chlorophyll	100	> 100	> 100
Saccharine	frond number	100	> 100	> 100
	frond area	100	> 100	> 100
	chlorophyll	100	> 100	> 100
Stevioside	frond number	100	> 100	> 100
	frond area	100	> 100	> 100
	chlorophyll	100	> 100	> 100

*The second test with sucralose was performed. The tested concentrations were: 0.1, 1, 5, 10, 100 and 500 mg/l; NOECs and LOECs – Dunnett's test, ANOVA ($P < 0.05$); EC_{50} – non-linear regression ($P < 0.05$) with their 95% confidence intervals

in more detail and try to detect the mechanism of action on the plant cell. It is possible that this mode of action is typical only of multicellular plants (*Lemna minor*), and not of the lower unicellular freshwater algae *Desmodesmus subspicatus* (Kobetičová *et al*. 2016). It was documented that increased sucralose concentrations in long-term treatment (more than 21 days) led to greater photosynthetic capacity ΦPSII together with higher carbon uptake (Amy-Sagers *et al*. 2017).

A hundred percent inhibition of duckweed growth can usually cause a disruption of chlorophyll content (Mocová & Gyömbérová 2015), but surprisingly, it was not observed in this experiment, notwithstanding the concentration of 500 mg/l caused 100% inhibition of plant growth at the morphological level. This indicates that sucralose apparently made the division of plant cells impossible, but did not cause the degradation of cells and did not affect existing photosynthetic pigments. This conclusion is in accordance with results of Amy-Sagers *et al.* (2017), where the authors described an increase of chlorophyll and sucralose metabolism. These authors suggested *L. minor* as a suitable model in bioremediation of PPCP from wastewaters.

CONCLUSIONS

The artificial and natural sweeteners investigated here showed various effects in the sub-chronic test with *Lemna minor* up to 500 mg/l. The results were expressed as dose-response curves, and for this reason the tested concentrations were higher than the present ecologically relevant levels. It is evident that some of the sweeteners (sucralose and aspartame) can affect the growth rate of this species in units of milligrams per litre or lower. The NOEC and LOEC values found in this work indicate a relatively higher risk potential for some sweeteners than the data on the other aquatic organisms indicate (Huggett & Stoddard 2011; Soh *et al.* 2011; Stolte *et al.* 2013, Hu *et al*. 2016; Amy-Sagers *et al*. 2017). These discrepancies might be explained by the use of different chemicals and their tested forms (pure chemicals versus tablets) and different design of the test performed in the present study and in the studies listed in the actual literature sources. In accordance with the latest studies (Hu *et al*. 2016; Amy-Sagers *et al.* 2017) it can be noted that *L. minor* is a suitable organism to be used for bioremediation purposes of the presumed non-degradable sweeteners. In this

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context obligatory ecotoxicity testing of environment samples and food additives would appear to be important.

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