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ORIGINAL PAPER

Occurrence and ecotoxicity of selected artificial sweeteners in the Brno city waste water

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Abstract

This study focused on the appearance of the most commonly used artificial sweeteners (acesulfame K, cyclamate, saccharin, and sucralose) in waste and discharged water and the evaluation of their possible negative effects on the water ecosystem. Samples of raw and treated wastewater were collected at the inflow and outflow of the Wastewater Treatment Plant in Brno-Modřice (Czech Republic). The target compounds were isolated by solid phase extraction and determined by liquid chromatography with mass spectrometric detection. The ecotoxicity of target compounds expressed as the E_rC_{20}, LC_{20}, and EC_{20} values was evaluated by a battery of tests using representatives of producers and consumers in the water ecosystem as testing organisms (*Lemna minor*, *Thamnocephalus platyurus*, and *Daphnia magna*). The results proved that the acute toxicity of individual artificial sweeteners does not pose a significant risk to the aquatic ecosystem. The compound showing the highest concentration in raw wastewater (in the range of tens of μg L^{-1}) and negligible removal during the wastewater treatment process was sucralose, but its ecotoxicity was the lowest of the ASs studied. The highest ecotoxicity showed saccharin, but its removal in a wastewater treatment plant was almost complete. However, additional studies are needed to evaluate the chronic toxicity of ASs and also possible synergistic or antagonistic effects in ASs mixtures.

Keywords Artificial sweeteners · Ecotoxicity · Liquid chromatography/mass spectrometry · Waste water

Introduction

Artificial sweeteners (ASs) are synthetically produced substances frequently used as low or zero caloric additives in non-alcoholic dietary beverages, in some cereal and dairy products and chewing gums (Sylvetsky and Rother 2016); they could be found in many pharmaceutical formulations and in personal care products such as tooth paste and mouthwash (Lin et al. 2017), and in animal feed (Buerge et al. 2011).

The oldest known artificial sweetener is saccharin, which was discovered in 1879 (Fahlberg and Remsen 1879). After its discovery, saccharin was used only by diabetics and later also by weight watchers. Its consumption grew substantially during World War I and World War II due to the shortage of sugar together with the logistic advantage given by its 300 × greater sweetness compared to sugar. Although its consumption declined between the world wars, after WWII it continued to grow, reaching a total of more than 159,000 tonnes in 2016 (Chemical Economics Handbook 2017). In the meantime, other ASs were discovered: cyclamate in 1937 (Lawrence 2003), aspartame in 1965 (Mazur et al. 1969), acesulfame in 1973 (Clauss and Jensen 1973), and sucralose in 1976 (Hough and Emsley 1986). Today, the highest use in food and beverage products is indicated for aspartame (18.5 thousand metric tons) followed by saccharin (9.7 thousand metric tons), acesulfame (6.8 thousand metric tons) and sucralose (3.3 thousand metric tons) (Euromonitor International 2017).

Despite the early marked entry and large-scale production of ASs, the information about their environmental fate is still limited (Luo et al. 2019). After ingestion, they are metabolized in the human body to a very limited extent or not at all, and after excretion in urine and faeces, they are transported via the sewage system to the wastewater treatment plant.

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The removal efficiency of current wastewater treatment technologies for individual artificial sweeteners is very different; saccharin is almost completely degraded, but acesulfame and sucralose pass the treatment processes almost unchanged (Buerge et al. 2009). Unremoved ASs residues are together with treated wastewater discharged to recipients.

The presence of sucralose in raw and treated wastewater at levels reaching 10 µg L^{-1} and in surface water in concentrations of an order of magnitude lower was reported for the first time in 2007 (Brorström-Lundén et al. 2008). Since then, a considerable number of publications have appeared that highlight the widespread distribution of artificial sweeteners in different environmental matrices (Kokotou et al. 2012; Luo et al. 2019). In untreated and treated wastewater, acesulfame and sucralose generally showed the highest concentrations (up to hundreds of µg L^{-1}) (Arbelaez et al. 2015). Acesulfame was also found in groundwater and drinking water (Ens et al. 2014) and together with saccharin and cyclamate in atmospheric precipitation (Gan et al. 2013). Sucralose was detected in coastal and open ocean waters (Mead et al. 2009). Acesulfame, saccharine, and cyclamate were found in the soil (Ma et al. 2017).

Studies dealing with the effect of ASs on aquatic organisms are still not very frequent. One of the first studies published in 2010 investigated the short-term effects of sucralose in two species of arctic copepods. Food intake was found to increase with increasing concentrations of sucralose in the marine copepod *Calanus glacialis* but not in *Calanus finmarchicus*, while egg production was not affected (Hjorth et al. 2010). Another study focused on the influence of sucralose on the growth of the duckweed plant *Lemna gibba*. After seven days test, no adverse effect was observed up to a sucralose concentration of 1000 mg L^{-1} (Soh et al. 2011). The duckweed plant *Lemna minor* was used as the testing organism for artificial sweeteners in concentrations of 6.25–100 mg L^{-1} in the 7-day test. Aspartame and sucralose inhibited growth parameters, acesulfame K did not cause significant negative effects, and saccharine showed slight stimulating effects (Kobeticova et al. 2018). The crustacean *Daphnia magna* after 4 h of exposure to sucralose (concentrations of 0.5, 5 and 500 µg L^{-1}) manifested a change in the behavioural response as altered swimming height and increased swimming speed (Wiklund et al. 2012). The No Observed Effect Level Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for acesulfame, cyclamate, saccharin, and sucralose were determined using the Reproduction inhibition assay with limnic green algae *Scenedesmus vacuolatus*, Acute immobilization assay with *Daphnia magna* and Growth inhibition assay with duckweed *Lemna minor*. For all compounds tested, the values of NOEC and LOEC were 1000 mg L^{-1} and > 1000 mg L^{-1}, respectively (Stolte et al. 2013). Another study presented NOEC and LOEC values of sucralose for *D. magna* as 1800 mg L^{-1} and > 1800 mg L^{-1}, respectively (Huggett and Stoddard 2011). Nevertheless, ASs could undergo transformation processes in environmental waters, and the products could exhibit elevated toxicity. The acute toxicity of ACE and SUC metabolites (after photo treatment) to *Vibrio fischeri* was found to be enhanced by factors 575 and 17.1, respectively (Sang et al. 2014). An increase in toxicity was also observed during the oxidative transformation of ACE by permanganate (Yin et al. 2017).

The bioconcentration test of sucralose at 10 and 100 mg L^{-1} on freshwater alga (*Pseudokirchneriella subcapitata*), crustacean (*Daphnia magna*) and zebrafish (*Danio rerio*) showed no bioaccumulation in these organisms (Lillicrap et al. 2011). On the contrary, sucralose bioconcentration in mammals (rat adipose tissue) has been reported (Bornemann et al. 2018). Acesulfame was also found to promote the uptake of Cd^{2+} by the green algae *Scenedesmus obliquus*, but simultaneously reduced its toxic effects (Hu et al. 2016).

The main goal of this study was: (1) to obtain information on the concentrations of frequently used ASs in raw and treated wastewater at the Municipal Wastewater Treatment Plant in the city of Brno, Czech Republic; (2) to evaluate the ASs removal efficiency of the wastewater treatment technology used in this facility; (3) to estimate the possible effects of ASs under study on the water biota. The study was carried out in April 2017.

Materials and methods

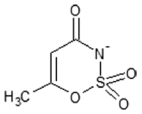
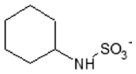
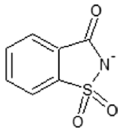
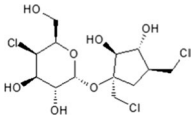
Chemicals

The list of target compounds, together with their relevant properties, is summarized in Table 1. Acesulfame-K, saccharin, and sucralose (purity ≥ 99%) were supplied by Sigma-Aldrich (Germany), cyclamate (purity ≥ 98.9%) was from Supelco (USA). Acetonitrile and methanol, both HPLC Gradient Grade, were from JT Baker (UK), hydrochloric acid (35%) was from Penta (Czech Republic), acetic acid (98%), formic acid (98%) and ammonium acetate (99%) were purchased from Sigma-Aldrich (Germany). Sucralose-d_6 (> 98%) used as surrogate was from Toronto Research Chemicals, Canada. Ultrapure water was obtained from a Milli-Q Academic water purification device (Merck Millipore).

Sampling and sample treatment

Wastewater samples were collected at the Wastewater Treatment Plant in Brno–Modřice, treating wastewater from the city of Brno and surrounding areas. This facility is equipped with two-stage treatment technology (mechanical

Table 1 List of target compounds and their properties

Compound	Acesulfame (ACE)	Cyclamate (CYC)	Saccharin (SAC)	Sucralose (SUC)
Empirical formula	$C_4H_4NO_4S^-$	$C_6H_{12}NO_3S^-$	$C_7H_5NO_3S^-$	$C_{12}H_{19}Cl_3O_8$
Structural formula				
Monoisotopic mass	161.9867	178.0543	182.1771	394.0353
Sugar equivalence values	200	30	300	600
Solubility in water [g L^{-1}]	270	1000	4	283
Human excretion [%]	100–101 [a]	> 99 [b]	90–100 [a]	> 95 [a]

[a]Values for unchanged parent compound (Mayer and Kemper 1991; Renwick 1985)

[b]Approximately 90% of the population excrete less than 1% of a daily dose of cyclamate as cyclohexylamine (Renwick 1986; Renwick et al. 2004)

and biological with anaerobic sludge stabilisation) and its capacity is 513,000 PE. The flow-proportional 24-h mixed samples were collected at the influent and effluent on April 10, 2017 (samples S1a and S1b), April 13, 2017 (samples S2a and S2b), and April 16, 2017 (samples S3a and S3b) using an automatic sampling device with a sampling interval of 2 h. The collected samples were transferred to pre-cleaned glass bottles, transported to the laboratory on ice, and stored in a refrigerator at 4 °C until analysis, which was performed within 24 h of collection. Each sample was analysed in triplicate using subsamples of 100 mL volume, which were filtered by a 0.22 µm membrane filter and acidified to pH 4 by hydrochloric acid. Before SPE, the samples were spiked with the internal standard sucralose-d_6.

Solid phase extraction

For solid phase extraction, cartridges Strata-X 200 mg/3 mL (Phenomenex) and Supelco VisiprepTM SPE Vacuum Manifold (Sigma-Aldrich) were used. The cartridges were conditioned with 3 × 3 mL of methanol and 3 × 3 mL of Milli-Q water acidified to pH 4 by HCl. The samples were passed through the activated cartridges at a flow rate of approximately 5 mL min^{-1} and then the columns were washed with 3 mL of Milli-Q water acidified to pH 4 by HCl. After washing, the columns were air-dried for 30 min. Elution was carried out with 3 × 3 mL of methanol and the extract was evaporated under a gentle stream of nitrogen at a temperature of 40 °C. The solid residue was dissolved in 1 mL of methanol/water mixture (1:1).

Liquid chromatography/mass spectrometry

LC/ESI–MS system was an Agilent 1100 Series HPLC coupled to a 6320 Series Ion Trap mass spectrometer with electrospray ionization (Agilent Technologies). Column Ascentis Express C18 (15 cm × 2.1 mm ID, 2.7 µm particles, Supelco) and a binary mobile phase consisting of methanol (solvent A) and Milli-Q water acidified by acetic acid to pH 4 (solvent B) were used for separation. The flow rate was set to 0.25 mL min^{-1}, the mobile phase gradient started from 10% of A and increased to 70% of A in 10 min, remained constant for 1 min, then returned to initial conditions in 1 min, and the last step was equilibration for 4 min. The column temperature was 25 °C, the injection volume was 2 µL. The nebulizer gas was nitrogen at a pressure of 172.4 kPa (25 psi), the drying gas (N_2) flow was 10 mL min^{-1}, and the drying temperature was 350 °C. Negative ions were scanned in the range of m/z from 50 to 600 u at a scan rate of 26,000 u sec^{-1}. ICC (Ion Charge Control) was switched on with a smart target value of 20,000 and the target ion was set to 350 u.

For identification and quantification of target compounds, the retention time matching on fragmentograms of characteristic m/z values was used. Acesulfame, saccharin, and cyclamate are anions, so they were detected at their monoisotopic masses (at m/z 162, 182, and 178, respectively), sucralose was detected as Cl^- adduct at m/z 433.

Ecotoxicity tests

To assess the impact of selected artificial sweeteners on the aquatic ecosystem, organisms representing both producers and consumers were used. The first group was represented by the aquatic plant *Lemna minor*, the second group included crustaceans *Thamnocephalus platyurus* and *Daphnia magna*.

Due to the expected low ecotoxicity of the compounds tested, the concentrations of ASs in the preliminary tests were 100, 300, 900 and 1800 mg L^{-1} for *L. minor* and 100, 200, 400, 800 and 1600 mg L^{-1} for *T. Platyurus* and *D. magna*. On the basis of the results obtained, it was decided

whether the selected concentration range would be sufficient, supplemented, or modified.

For comparison, the natural sweetener licorice was also subjected to ecotoxicity tests.

Duckweed growth inhibition test

On monocotyledonous aquatic plant *Lemna minor* as representative of producers was performed according to the OECD Test No. 221: *Lemna* sp. Growth Inhibition Test (OECD 2006). A laboratory stock culture of *L. minor* was used. Seven days before testing, sufficient colonies were transferred aseptically into fresh sterile modified Steinberg medium (ISO 2005) and cultured under the test conditions (the chamber was continuously illuminated to a maximum of 7 100 lx). No contaminating organisms (such as algae) were present. The tests were performed in duplicate. Beakers of 150 ml volume were filled with 75 mL of ASs solutions of selected concentration range and with modified SM as the control. The test plants were then transferred to each beaker so that the number of fronds in each beaker was 9, the beakers were covered, and the plants were exposed to a constant white light top illumination of 7000 lx provided by CW fluorescent light (F36W/33–640, Sylvania) at a constant ambient temperature of 22 °C for 7 days. After this period, the fronds were counted and the average specific growth rate was evaluated using the equation:

$$\mu = \frac{\ln\left(N_7\right) - \ln\left(N_0\right)}{t}$$

where μ is the average specific growth rate, N_0 and N_7 are the numbers of fronds observed at the beginning and at the end of the test, and t is the test duration.

Inhibition of the growth rate was then evaluated as

$$I_r[\%] = 100 \times \frac{\mu_C - \mu_T}{\mu_C}$$

where I_r is the inhibition of the average specific growth rate, μ_C is the mean value of the specific growth rate in the control, and μ_T is the mean value of that in the treatment group. From the so-called inhibition equation, the values of $168hE_rC_{20}$ were calculated. The test results were considered valid if (ISO 2005):

(1) the average number of leaves in the control has increased eightfold,
(2) the pH in the control sample has not changed by more than 1.5 units and
(3) the $168hIC_{50}$ value of potassium dichromate was between 5.5 and 10 mg L^{-1}.

Alternative toxicity test on the organism *Thamnocephalus platyurus*

(Microbiotests Inc, Gent, Belgium) has been performed as a 24-h acute toxicity test. Testing was carried out according to the Thamnotoxkit F™ Standard operational procedure manual for the microbiotest toxkit, whose methodology complies with the ISO 14380 standard (ISO 2011). Concentrated solutions of $NaHCO_3$, $CaSO_4$, $MgSO_4$, KCl (incorporated in the toxkit), and Milli-Q water were used to prepare one litre of Standard Freshwater (SF), which was used for cyst hatching and as the toxicant dilution medium. The organisms were activated by hydration in diluted SF medium (dilution 1:8 with Milli-Q water) and stored in an incubator at a constant temperature of 25 °C and continuous illumination of 4000 lx. The wells in a 24-well plate were filled with 1 mL of the control (SF) or tested solution and subsequently 10 hatched larvae were transferred to each well. The plate was then covered with a transparent lid, which was sealed with a parafilm strip and incubated in the dark at 25 °C for 24 h. The tests were performed in triplicate. Mortality (number of dead organisms) was determined in individual wells and used to calculate the $24hLC_{20}$ and/or $24hLC_{50}$ values.

Alternative toxicity test on *Daphnia magna*

(Microbiotests Inc, Gent, Belgium) was performed as an acute toxicity test of 24 and 48 h according to the Standard Operation Procedure Manual of the microbiotest toxkit Daphtoxkit F™, whose methodology corresponds to the EN ISO 6341 standard and the OECD Guideline 202 (OECD 2004). Standard Freshwater was prepared from stock solutions included in the toxkit ($NaHCO_3$, $CaCl_2$, $MgSO_4$, and KCl) by dilution with Milli-Q water and used for hatching of the Ephippia (dormant eggs) and for preparation of concentration series of tested ASs. After hatching (light of 6000 lx, temperature 25 °C, and duration 75 h), the organisms were prefed with Spirulina algae to prevent unsolicited mortality within the next 48 h. After filling the 30-well test plate wells with 10 mL of toxicant dilution series and SF as control, 10 test organisms were placed into each well and after covering the plate with a lid and sealing with a parafilm strip, incubation was carried out in darkness at 20 °C. Four parallel tests were performed for all concentration levels and test durations.

After 24 and 48 h of incubation, mortality (number of dead daphnia) and immobilization (number of daphnia that cannot swim after gentle agitation of the liquid for 15 s even if they can still move their antennae) were evaluated as the test endpoints. Based on the data obtained, the values of $24hLC_{20}$, $24hLC_{50}$, $48hLC_{20}$, and $48hLC_{50}$ were calculated.

Statistical analysis

Regression and statistical analyses were performed using GraphPad Prism 9.1 (GraphPad Software). Statistical differences between groups ($p < 0.05$) were analysed using Student *t*-test with Welch's correction applied in the cases where the *F*-test identified significant differences in group variance.

Results and discussion

Concentrations of artificial sweeteners in waste water

Samples of raw and treated wastewater were taken from the Brno-Modřice Wastewater Treatment Plant. At the inflow, the highest concentrations were shown by sucralose (12–23 μg L^{-1}), followed by saccharin (14–20 μg L^{-1}). The levels of acesuflame-K and cyclamate were one order of magnitude lower. SUC as the most abundant artificial sweetener in waste water has been reported quite rarely (e.g. Van Stempvoort et al. 2011), but the order of ASs' concentrations is usually different; e.g. influent concentrations two WWTPs in Germany for ACE and SAC ranged between 34 and 50 μg L^{-1}, CYC level was up to 190 μg L^{-1} and SUC concentration was below 1 μg L^{-1} (Scheurer et al. 2009). Similar results were found in raw wastewater in northwest Spain (CYC 26–36 μg L^{-1}, ACE 25–27 μg L^{-1}, SAC 18–22 μg L^{-1}, SUC 3–5.3 μg L^{-1}) (Ordóñez et al. 2012). Interestingly, effluent concentrations of ACE (31–33 μg L^{-1}) and SUC (16–18 μg L^{-1}) were higher than those in the influent. This could be attributed to the release of adsorbed SUC on the suspended particulates from influents after the treatment in WWTPs (Subedi and Kannan 2014). A similar effect was also observed in Switzerland in 10 WWTPs, where influent ACE levels were 12–43 μg L^{-1} and elimination efficiency was $-9 \pm 28\%$ (Mean ± SD); for SUC these values were 2.0–9.1 μg.L^{-1} and $-5 \pm 13\%$. The influent levels of CYC and SAC were 15–65 and 3.9–18 μg L^{-1}; both compounds were effectively removed with elimination efficiencies of 99 ± 1 and $90 \pm 14\%$, respectively (Buerge et al. 2009).

In the present work, differences in SUC inflow and outflow concentrations were not statistically significant (two-sample *t*-test, $\alpha = 0.05$). Other ASs under study were removed almost completely.

The concentrations of the target compounds in the collected samples are summarized in Table 2.

Ecotoxicity tests of sweeteners

Lemna minor growth inhibition test

The validity of the test was confirmed by meeting the criteria defined by the OECD Guidelines 221 (OECD 2006), requesting a doubling time of the number of fronds less than 60 h and an average specific growth rate of 0.275 d^{-1}. Our results (in the worst case) were 55 h and 0.299 d^{-1}, respectively.

The results of the ecotoxicological tests of selected ASs on *L. minor* are shown in Fig. 1, Table 3, and in Tables S1–S4 in Supporting Information.

The values of 168hIC_{20} and 168hIC_{50} could only be determined for saccharin and licorice. For acesulfame-K and sucralose, these values could not be evaluated because duckweed growth was inhibited only at the highest concentration

Table 2 Results of waste water analysis

Sampling		April 10, 2017		April 13, 2017		April 16, 2017	
		Concentration [μg L^{-1}]	RE [%]	Concentration [μg L^{-1}]	RE [%]	Concentration [μg L^{-1}]	RE [%]
ACE	*In*	2.071 ± 1.071	–	1.041 ± 0.744	–	1.535 ± 0.421	–
	Out	< LOD		< LOD		< LOD	
CYC	*In*	4.049 ± 0.682	–	4.110 ± 0.853	–	2.894 ± 0.392	–
	Out	< LOD		< LOD		< LOD	
SAC	*In*	19.55 ± 7.323	93.5	13.62 ± 1.227	–	18.62 ± 0.515	97.6
	Out	1.266 ± 0.816		< LOD		0.455 ± 0.153	
SUC	*In*	22.67 ± 2.683*	2.16	20.92 ± 5.234*	2.37	12.23 ± 1.488*	17.6
	Out	22.18 ± 1.005*		20.42 ± 2.669*		10.08 ± 1.432*	

In … concentration at inflow, *Out* … concentration at outflow. Concentrations are presented as the mean for 3 replicates ± standard deviation

ACE Acesulfame-K; *CYC* Cyclamate; *SAC* Saccharin; *SUC* Sucralose

RE—Removal Efficiency, $RE[\%] = 100 \times \frac{c_{input} - c_{output}}{c_{input}}$

* … differences between inflow and outflow concentrations are not statistically significant (Student *t*-test, $\alpha = 0.05$)

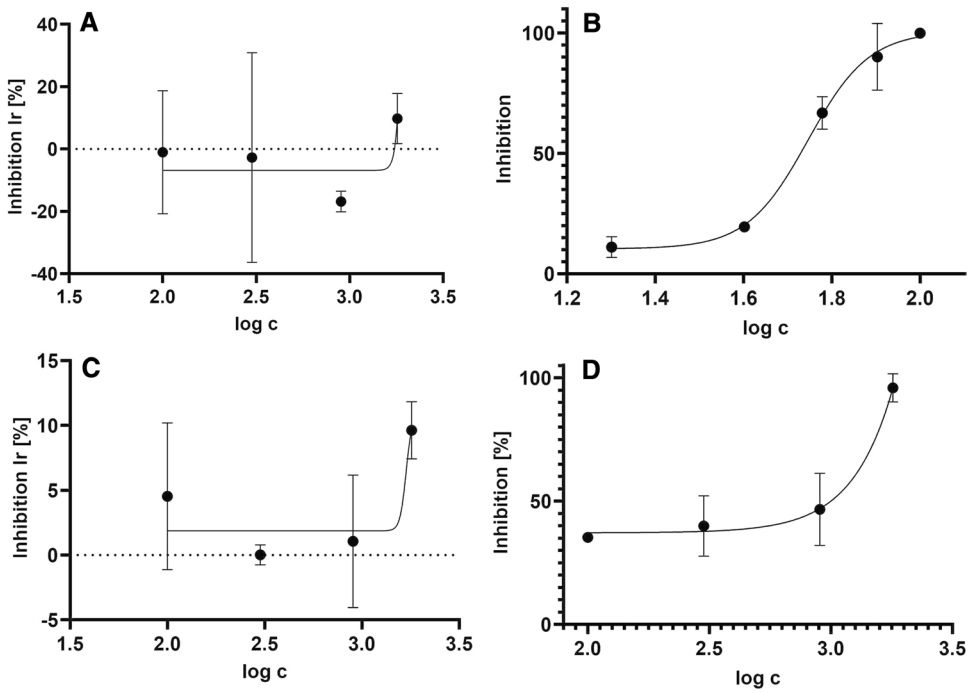


Fig. 1 Results of ecotoxicity testing on *L. minor*. Panel A: acesulfame-K; panel B: saccharin; panel C: sucralose; panel D: licorice

Table 3 Results of ASs ecotoxicity testing on *L. minor*

Compound	168hIC$_{20}$ [mg L^{-1}]	168hIC$_{50}$ [mg L^{-1}]
Acesulfame-K	> 1800	> 1800
Saccharin	45	57
Sucralose	> 1800	> 1800
Licorice	956	1189

tested of 1,800 mg L^{-1}. Acesulfame at concentrations of 300 and 900 mg L^{-1} even stimulated duckweed growth.

Alternative toxicity test on *Thamnocephalus platyurus*

The validity of the test was verified according to the procedure given in ISO 14380:2011 (ISO 2011) using solutions of potassium dichromate. Results are presented in Fig. 2, Panel A, and in Table S5 in Supporting Information.

According to ISO 14380, the test is valid if the 24hLC$_{50}$ value is in the range of 0.052–0.148 mg L^{-1}. Our result was 0.0748 mg L^{-1}, which is within the defined range.

The results of the ecotoxicological testing of selected AS on *T. platyurus* are presented in Fig. 2 Panels B—F and Table 4. Measured data are presented in Tables S5–S10 in Supporting Information.

The results of ecotoxicological tests of selected artificial sweeteners on *T. platyurus* are summarized in Table 4.

From the dose–response curves in Fig. 3, it follows that the values of 24hLC$_{20}$ and 24hLC$_{50}$ can be reliably determined only for saccharin, whose curve showed the usual sigmoidal shape, and with less reliability also for acesulfame-K, where the average morality at the highest concentration tested is close to 100%. The remaining two sweeteners showed at the highest concentrations tested an average mortality of up to 40%, which only allowed the estimation of 24hLC$_{20}$.

Alternative toxicity test on *Daphnia magna*

The validity of the test was verified according to EN ISO 6341 (ISO 2012) using a potassium dichromate solution. Results are summarized in Fig. 3 and Table S11 in Supporting Information.

According to EN ISO 6341 (ISO 2012), the test is valid if the value of 24hEC$_{50}$ is between 0.6 and 2.1 mg L^{-1}, which is met. The 24hEC$_{50}$ and 48hEC$_{50}$ declared by the manufacturer were 1.32 mg L^{-1} and 0.84 mg L^{-1}, respectively; our results agree very well with these values. Therefore, the results of the Alternative toxicity test on *Daphnia magna* are valid.

The test results of the target ASs are presented in Fig. 4 and Table 5. Measured values are summarized in Tables S12-S16 in Supporting Information.

According to published data, artificial sweeteners exhibit only low acute toxicity and negative effects were observed only at higher concentrations (Huggett and Stoddard 2011; Tollefsen et al. 2012). However, these concentrations would not be relevant with respect to the observed environmental levels.

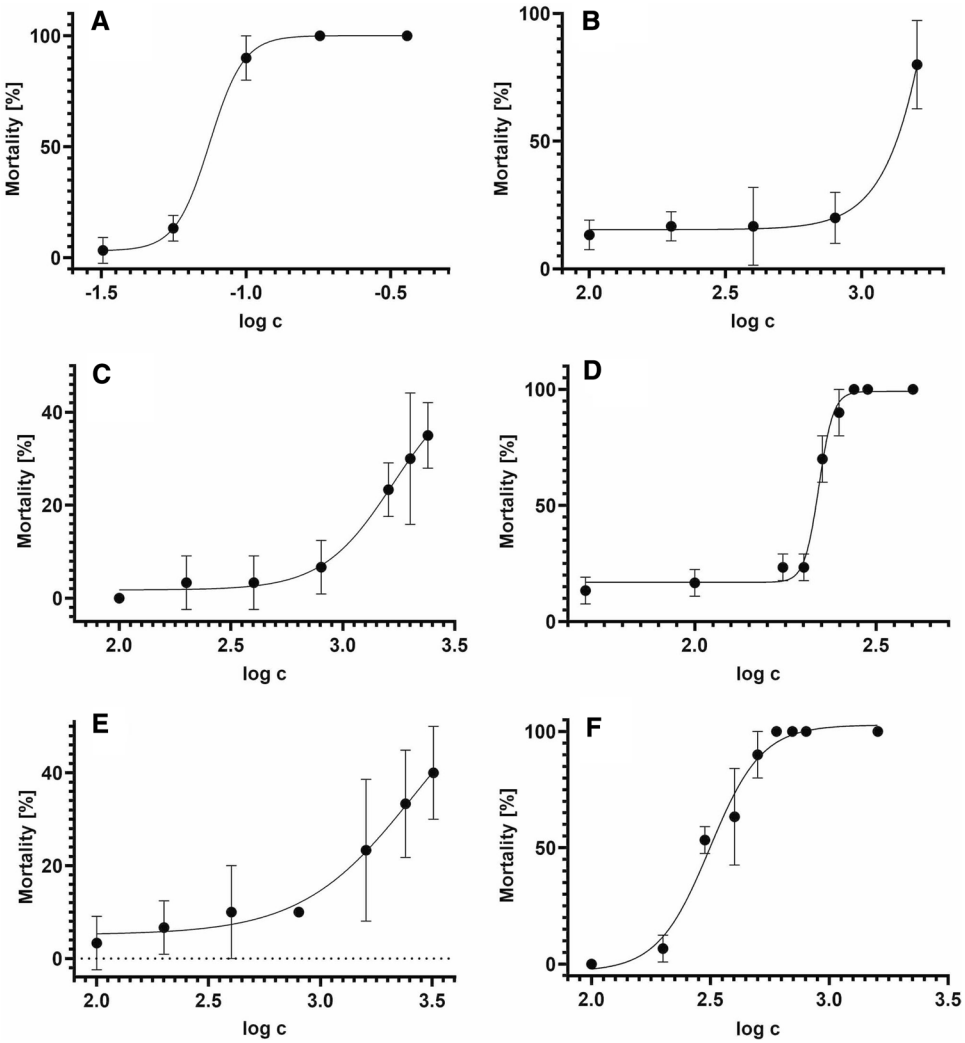


Fig. 2 Results of alternative toxicity tests on *T. platyurus*. Panel A: validity test; panel B: acesulfame-K; panel C: cyclamate; panel D: saccharin; panel E: sucralose; panel F: licorice

Table 4 Results of ASs ecotoxicity testing on *Thamnocephalus platyurus*

Compound	$24hLC_{20}$ [mg L^{-1}]	$24hLC_{50}$ [mg L^{-1}]
Acesulfame-K	1032	1426
Cyclamate	1520	> 2400
Saccharin	206	219
Sucralose	1645	> 3200
Licorice	221	314

In the case of sucralose testing on *D. magna*, after 24 h at the highest tested concentration (1600 mg L^{-1}) an average mortality of 20% was observed and after 48 h this value increased only to 25%. Acesulfame-K even stimulated the growth of *L. minor*, for sucralose only a small inhibition was observed compared to that of the control.

The values of $168EC_{20}$ for *L. minor*, $24hLC_{20}$ for *T. platyurus*, $24EC_{20}$ and $48EC_{20}$ for *D. magna* are summarized in Fig. 5.

On the basis of the results of all conducted tests, a similar trend in sweeteners ecotoxicity for all testing organisms was observed. The most toxic effects were exhibited by saccharin and liquorice. To our knowledge, the ecotoxicological evaluation of liquorice has not been published till now; the only natural sweetener evaluated in this field is stevioside, which exhibited only low risk potential for aquatic organism (Stolte et al. 2013).

Surprisingly, the ecotoxicity obtained in the present study for saccharin is different from the published data. In the study of Stolte et al. (2013), saccharin did not affect the test organisms (*L. minor, D. magna*) up to the concentration of 1000 mg L^{-1}, but in this study inhibition was already observed at a concentration of 45 mg L^{-1} for *L. minor* and of 150 mg L^{-1} for *D. magna*. In the study mentioned, the ecotoxicity of high-intensity sweeteners ACE, CYC, and SAC on *D. magna*, *L. minor,* and *Scenedesmus vacuolatus* organisms was evaluated. The determined NOEC value for all sweeteners was 1000 mg L^{-1}. In this study, the $24hLC_{20}$ and $48hEC_{20}$ values for ACE and CYC were found to be around

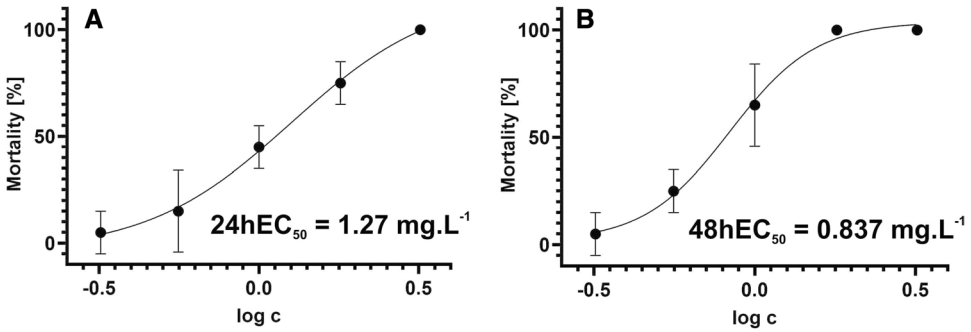


Fig. 3 Validity verification of Alternative toxicity test on *Daphnia magna* with potassium dichromate solution. Panel A: 24 h test, panel B: 48 h test

1000 mg L^{-1}, which means that the NOEC values must be lower. Furthermore, the ecotoxicity of saccharin was higher in our study for all testing organisms; the adverse effect was already observed at a concentration of 100 mg L^{-1}, 100% mortality was observed at a concentration of 400 mg L^{-1}.

Stolte et al. (2013) tested AS ecotoxicology on the green algae *S. vacuolatus, which* plays an important role in the ecosystem. None of the investigated sweeteners showed statistically significant adverse effects in test concentrations up to 1000 mg L^{-1}.

By testing the semi-chronic toxicity of sucralose to *L. gibba*, the study (Soh et al. 2011) found that no adverse effect was observed up to the concentration of 1000 mg L^{-1}. Similar conclusions were also found in our study: during sucralose tests on *L. minor*, inhibition of the plant at the highest concentration (1800 mg L^{-1}) was only 10.81%.

In Fig. 1 panel A, the so-called hormesis effect of acesulfame-K on *L. minor* could be observed. This is the stimulation of growth at lower concentrations of the compound tested, resulting in negative inhibition values. Hormesis is induced by activation of the defence mechanisms of tested organisms at low concentrations of toxic substances (Calabrese and Baldwin 2002; Kefford et al. 2008) and is quite common (Calabrese and Baldwin 2001; Calabrese and Blain 2005). In the present work, stimulation was observed up to a concentration of 900 mg L^{-1}. However, our test lasted for seven days and the question is whether the hormesis would be observable after a longer time, because it is possible that this substance really has a stimulating effect.

Currently, there is a lack of studies that focus on the acute or chronic toxicity of artificial sweeteners. Furthermore, other endpoints other than the mortality of the organism after exposure to the substance were very rarely evaluated. For complex substance evaluation, there is a need to investigate chronic toxicity and physiological changes at suborganismal levels. The next step towards a comprehensive assessment of artificial sweeteners in the environment would be the implementation of chronic toxicity studies using low doses for long periods. This arrangement closely reflects the current situation in the environment, and so the ecotoxicological effects could be more accurately determined.

It should also be kept in mind that artificial high-intensity sweeteners are not the only substances incompletely removed in WWTP. Consequently, attention should also be paid to testing mixtures in which artificial sweeteners can exhibit a synergistic effect.

Conclusion

The aim of the study was to analyse artificial sweeteners in the influent and effluent of WWTP Brno-Modřice and to carry out ecotoxicological tests on representatives of aquatic environment organisms to estimate the effect of ASs on the water biota.

Three 24-h mixed samples collected at the inflow and outflow of the WWTP Brno-Modřice (Czech Republic) were treated by Solid Phase Extraction and analysed by liquid chromatography with mass spectrometric detection using electrospray ionization. All aforementioned sweeteners were determined in the influent at concentrations ranging from 1.40 (acesulfame-K) to 22.67 µg L^{-1} (sucralose). The concentrations of cyclamate and saccharin were 4.05 µg L^{-1} and 19.55 µg L^{-1}, respectively. By effluent water analysis, it was found that only about 6% of sucralose was removed during treatment. The removal efficiency of saccharin was about 95%. ACE and CYC at the outflow were below the limit of quantification.

The possible impact of selected artificial high intensity sweeteners on aquatic organisms was evaluated by ecotoxicological tests using freshwater crustaceans *T. platyurus* and *D. magna* and aquatic plant *L. minor*. Saccharin was found to be the most toxic, with a value of $24hLC_{20}$ for *T. platyurus* established at 206 mg L^{-1}, $48hEC_{20}$ for the organism *D. magna* was 150 mg L^{-1} and $168hEC_{20}$ for *L. minor* was 45 mg L^{-1}. Similar results were also obtained for licorice (a natural sweetener that was only tested to supplement the findings).

The results obtained showed that the acute ecotoxicity of artificial sweeteners is low and the observed harmful effects occurred only at concentrations that were much higher than those in recipient waters. Although artificial

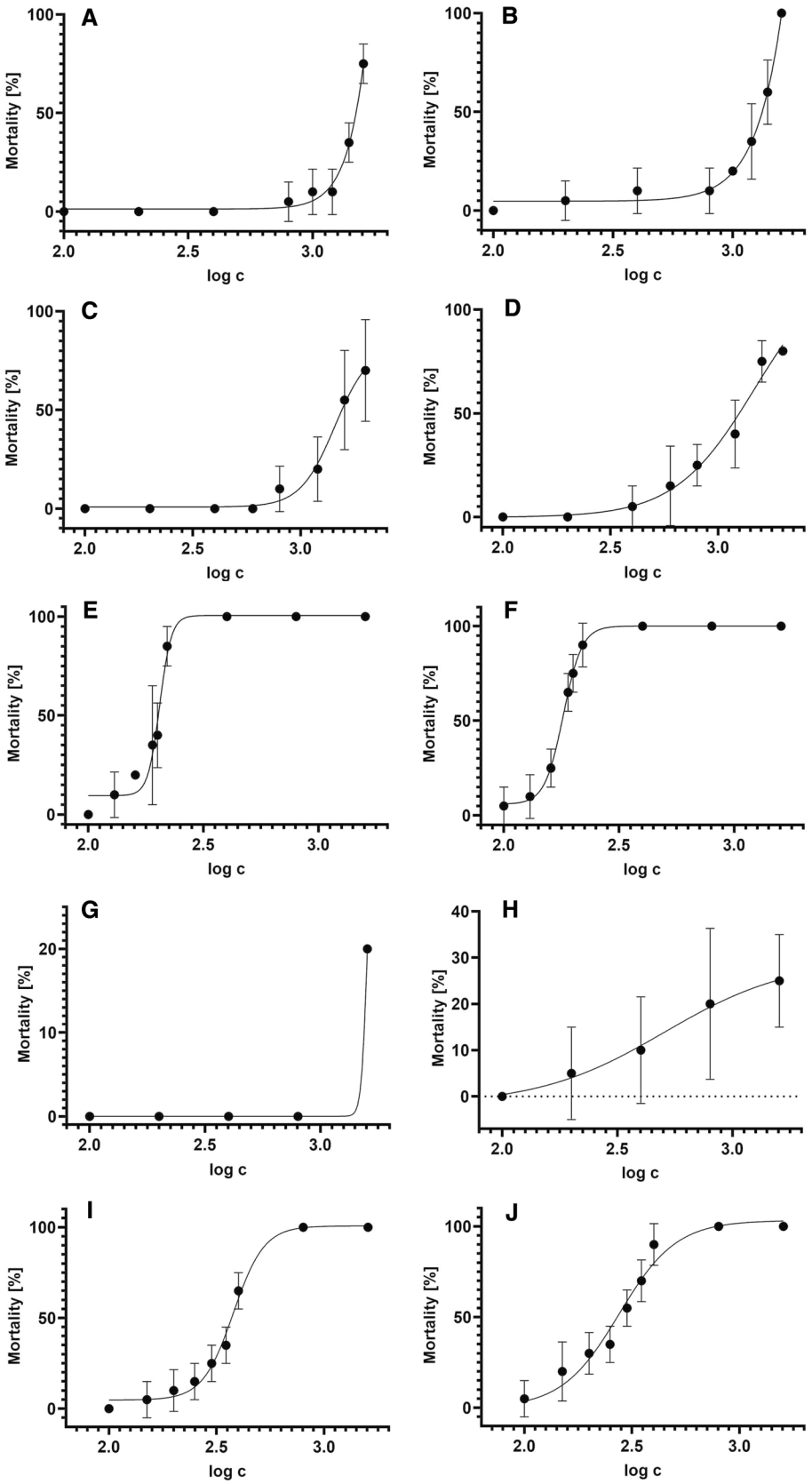


Fig. 4 Results of Alternative toxicity tests on *D. magna*. Panel A: acesulfame-K, 24 h test; panel B: acesulfame-K, 48 h test; panel C: cyclamate, 24 h; panel D: cyclamate, 48 h; panel E: saccharin, 24 h; panel F: saccharin, 48 h; panel G: sucralose, 24 h; panel H: sucralose, 48 h; panel I: licoricce, 24 h; panel J: licorice, 48 h

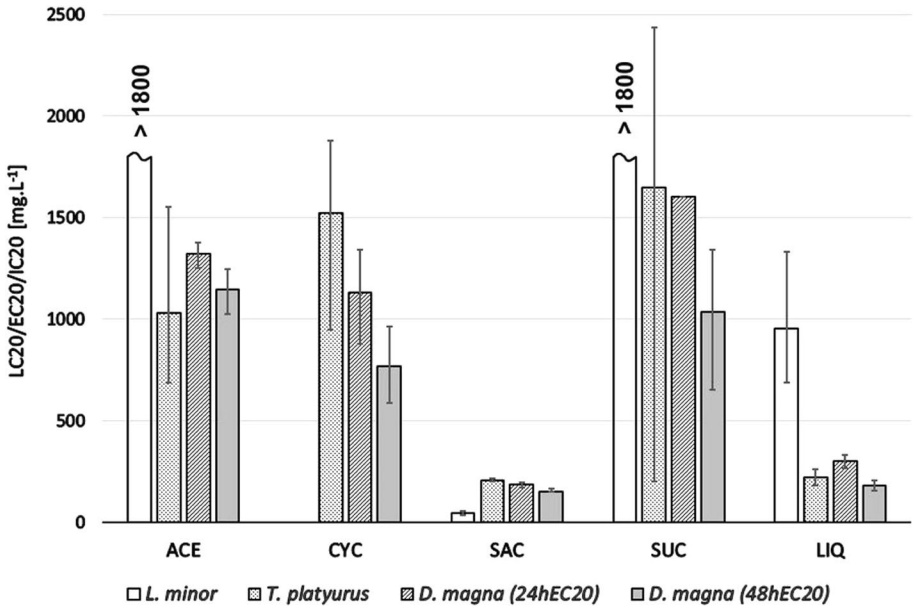


Fig. 5 Overview of the LC_{20}/EC_{20}/IC_{20} values of ASs tested. ACE—acesulfame-K; CYC—cyclamate; SAC—saccharin; SUC—sucralose; LIQ—liquorice. Confidence intervals at the 95% level were indicated

Table 5 Results of ASs ecotoxicity testing on *Daphnia magna*

Compound	24hEC_{20}	24hEC_{50}	48hEC_{20}	48hEC_{50}
Acesulfame-K	1321	1496	1143	1345
Cyclamate	1130	1585	770	1258
Saccharin	188	205	150	182
Sucralose	1600	1865	1034	8415
Licorice	302	377	180	286

sweeteners are not expected to represent an increased risk of acute toxicity for aquatic organisms, further tests and studies focused on chronic toxicity will be desirable. It will be useful to examine not only individual sweeteners but also their mixtures to evaluate possible synergic or antagonistic effects. Finally, the right choice of endpoints is also of great importance.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13762-021-03771-8.

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Availability of data and material All data are available either in publication or in Supporting Information.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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