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RESEARCH ARTICLE

Effect of eight common Brazilian drugs on *Lemna minor* and *Salvinia auriculata* growth

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Abstract

The growth of two species of macrophytes (*Lemna minor* and *Salvinia auriculata*) under the effect of a mixture of amoxicillin, caffeine, carbamazepine, dipyrone, ibuprofen, losartan, omeprazole, and tenivastatin was investigated by bioassay. Three concentration levels were utilized in this study (10, 200, and 500 μg L^{-1}) using a growth inhibition test based on the OECD 221/2006 guidelines. The frond number, total area, and chlorophyll *a* level were selected as suitable end points. For *L. minor*, at all concentrations, a significant difference in the total frond number was observed and the growth inhibition varied from 30 to 70% at the low and high concentrations, respectively. No significant growth change was observed to *S. auriculata* exposed to the mixture of drugs. Thus, individual drug tests were performed for *L. minor* which demonstrated stimulation in growth, when exposed to most drugs individually, except tenivastatin which was identified as the drug responsible for the significant growth inhibition seen in the mixture. The *L. minor* enhanced growth was probably caused by N molecule transformation to ammonium and nitrate, essential nutrients for plants.

Keywords Bioassay · Emerging contaminants · Macrophytes · Pharmaceuticals · Growth inhibition

Introduction

Maintaining water quality is one of the major concerns and challenges of the twenty-first century. One of the seventeen United Nations Sustainable Development Goals adopted for the 2030 Agenda is to ensure the sustainable management and availability of water and sanitation for the entire population (UN 2019).

Across Latin America, Asia, and Africa, one in seven rivers has been adversely affected by organic pollutants (UNEP 2016). A wide range of these compounds are discharged into the water environment every day, including industrial chemicals (i.e., polycyclic aromatic hydrocarbons, endocrine disruptors chemicals, and others), pesticides, personal care products, and drugs through domestic and industrial wastewater, either as raw effluent or with only a low level of treatment (Santos et al. 2010; Rodríguez-Rodríguez et al. 2011; Aguirre-Martinez et al. 2015; Aznar et al. 2017; Coelho et al. 2020). The degradation of these pollutants can, for example, cause a reduction in the dissolved oxygen present in water, unbalancing the aquatic ecosystem (UNEP 2016), or cause specific effects such as feminization in fish (Sanchez et al. 2012) or toxicity to non-target organisms (Escher et al. 2011; González-Pleiter et al. 2013; Orias and Perrodin 2013; Alkimin et al. 2019; Obinna and Ebere 2019).

Guidelines for chemical, toxicological, and ecotoxicological tests as tools to monitor aquatic ecosystems are continuously standardize by regulatory agencies, such as Organization for Economic Co-operation and Development (OECD), American Society for Testing and Materials (ASTM), International Organization for Standardization

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(ISO), United States Environmental Protection Agency (USEPA), European Environment Agency (EEA), and others. However, no legislative limits for most contaminants of emerging concern (drugs, endocrine disruptors, anthropogenic markers, and others) have been approved so far. Despite studies in different countries reporting the presence of a wide variety of emerging contaminants in different environmental compartments, no accurate data was gathered to quantify the chemical contamination profile (Tundisi and Tundisi 2008; Tundisi 2014; UNEP 2016).

While several studies have assessed the toxic potential of these pollutants against standard test organisms (e.g., algae, *Daphnia* spp., and fish), few have tested effects on aquatic macrophytes exposed to anthropogenic discharges (Khetan and Collins 2007; Carvalho et al. 2010; Connon et al. 2012; Nunes et al. 2014; Godoy et al. 2015; Noguera-Oviedo and Aga 2016). Several pharmaceutical compounds have been shown to have the capacity to be transferred from sediment or water and be absorbed by the roots of plants, accumulating in their leaves, stem, and fruits of edible plants such as cabbages, peas, and cucumbers (Herklotz et al. 2010; Tanoue et al. 2012; Nunes et al. 2014). Cereal plants such as wheat and barley and root vegetables such as carrots have presented similar results (Trine et al. 2011). Additionally, drugs were detected in roots, stems, leaves, and seeds of plants growing in biosolid treated soil (Wu et al. 2010). The effect of plant uptake is not fully understood and depends on the properties of the pharmaceutical compound, the physical characteristics, and species of the plants (Carvalho et al. 2010; Tanoue et al. 2012; Jakimska et al. 2014; Taylor-Smith 2015). Floating macrophytes are even used for phytoremediation due to their ability of absorption (Carvalho et al. 2010).

This study exposed macrophytes to a mixture of pharmaceuticals widely used in human and veterinary medicine in Brazil and frequently detected in Brazilian waters, which comprises amoxicillin (antibiotic), caffeine (stimulant), carbamazepine (antiepileptic), dipyrone (analgesic), ibuprofen (anti-inflammatory), losartan (antihypertensive), omeprazole (digestive), and tenivastatin (cholesterol control) (Stumpf et al. 1999; Almeida 2003; Ghiselli 2006; Sodré et al. 2007; Costa 2009; Montagner and Jardim 2011; Américo et al. 2012; Thomas et al. 2014; Shihomatsu et al. 2015; Bertoldi et al. 2016). Thus, they have the potential for constant released into the environment, via treated sewage effluent from wastewater treatment plants (WWTPs) and raw sewage from irregular (unauthorized) homes or from livestock and agriculture (Aznar et al. 2017).

Lemna minor and *Salvinia auriculata* are aquatic plants, well known as ecological indicators, since their growth rate can be affected by various classes of pollutants. *L. minor* is the standard aquatic freshwater plant in many tests (i.e., OECD); recently, *L. minor* has been used to assess the environmental risk of emerging contaminants such as drugs (Fekete-Kertész et al. 2015; Godoy et al. 2015; Aznar et al. 2017). *S. auriculata* is native to tropical waters such as in Brazil and this macrophyte is also considered a good bioindicator with high growth rate and sensitivity to toxic agents such as metals. However, the effect of emerging pollutants at environmental concentrations (10 μg L^{-1}) on these plants has been poorly investigated.

L. minor has simple structure and morphology with rapid growth rate, whereas *S. auriculata* has floating and submerged leaves, the latter works like roots absorbing water and ions (Santos et al. 2020).

The main hypothesis tested in this paper is that the growth of *L. minor* and *S. auriculata* is affected when exposed to a single drug or a mixture of drugs and thus these can be utilized as potential indicators of drug presence in the environment or as suitable as test organisms in standardized evaluation protocols. Thus, the main objective of the present work was to evaluate the influence of eight selected drugs classified as contaminants of emerging concern (amoxicillin, caffeine, carbamazepine, dipyrone, ibuprofen, losartan, omeprazole, and tenivastatin) on the growth of aquatic plants (*L. minor* and *S. auriculata*) under laboratory conditions.

Material and methods

Chemicals and reagents

Analytical standard of amoxicillin (AMO), caffeine (CAF), carbamazepine (CBZ), dipyrone (DIP), ibuprofen (IBU), losartan (LOS), omeprazole (OME), and tenivastatin (TEN) were purchased from Sigma-Aldrich. HPLC grade methanol was obtained from Merck. *L. minor* was obtained from a natural pond in Scotland and stock cultures of the healthy fronds were maintained in Steinberg medium for 1 week before commencing the test. *S. auriculata* was obtained from Tropica Aquarium Plants, Aarhus, Denmark, and maintained in stock cultures using the same media.

Tenivastatin is an active metabolite of simvastatin which readily breaks down when in contact with water. Hence, tenivastatin was used in preference as this form would be most likely under aqueous conditions.

Chemicals used in Steinberg medium (OECD 2006) are described in Table 1 and purchased from Sigma-Aldrich.

Pharmaceuticals effect on plant growth

Two experiments were planned to identify the effect of 8 drugs on the growth of *L. minor* and *S. auriculata*. The first experiment (Test 1) exposed the two plant species to a mixture of 8 drugs at two concentrations and that was repeated three times, with an incubation period of 7 days (details in "Mixed drug growth test" section). After this test, the assessment of the effects of individual drugs (Test 2) was performed for the species that showed

Table 1 Steinberg solution composition

Stock solution	Composition	g L^{-1}	Aliquot for 1 L (mL)
1	KNO_3	17.50	20
	KH_2PO_4	4.5	
	K_2HPO_4	0.63	
2	$MgSO_4 \cdot 7H_2O$	5.00	20
3	$Ca(NO_3)_2 \cdot 4H_2O$	14.75	20
4	H_3BO_3	0.120	1
5	$ZnSO_4 \cdot 7H_2O$	0.180	1
6	$Na_2MoO_4 \cdot 2H_2O$	0.044	1
7	$MnCl_2 \cdot 4H_2O$	0.180	1
8	$FeCl_3 \cdot 6H_2O$	0.760	1
	EDTA disodium-dihydrate	1.500	

impacted growth. A flow chart describing Test 1 ("Mixed drug growth test" section) and Test 2 ("Individual drug test on the *L. minor* growth" section) is presented in Fig. 1.

Mixed drug growth test

All tests were setup according to the OECD 221 (2006) guideline, in translucent glass tanks with a capacity of approximately 2.5 L. The tanks surface was illuminated, at a rate of 6500 to 10,000 lux by 4 fluorescent lamps programmed for a photo period of 16 h light and 8 h in the dark.

The drugs used were initially evaluated as two separate mixtures in which the eight drugs were present, individually, at concentration of 10 or 500 μg L^{-1}. The tests were established for *L. minor* and *S. auriculata*, utilizing 8 tanks for each plant, 2 replicate tanks with no drugs added acted as the control, 3 replicate tanks for the drug mixture at a concentration of 10 μg L^{-1}, and 3 replicate tanks for the mixture at a concentration of 500 μg L^{-1}.

Each tank was filled with 2 L of Steinberg medium containing the appropriate concentrations of the 8 drugs. For *L. minor*, 33 fronds were carefully placed on the surface of the tank, whereas for *S. auriculata*, only 24 fronds were used. The temperature was kept around 20 °C and pH was recorded at the beginning and when the drugs exposure ended.

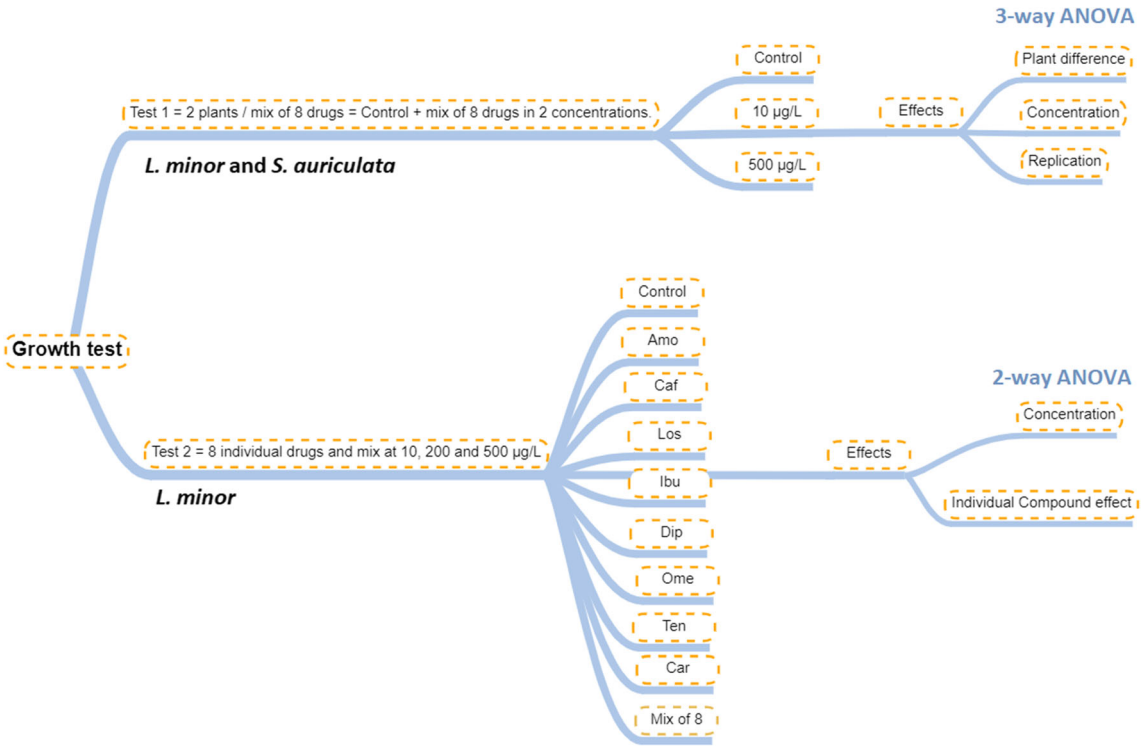

Fig. 1 Experimental design for the evaluation of 8 drugs effect on two macrophytes (*Lemna minor* and *Salvinia auriculata*)

The variables chosen as test end points were based on OECD guideline being frond number (FN), total area (TA), and chlorophyll *a*. The FN was recorded at the beginning and after 7 days. For TA, the fronds were collected at the end of test, dried slightly with absorbent paper, and scanned to calculate overall area with Black Spot Leaf Area Calculator software version 1.0 beta (Bangalore, India).

Chlorophyll *a* analysis was performed for each replicate according to the procedure described by Sumanta et al. (2014), when the test ended. All fronds from each replicate were transferred to a 2 mL tube containing 6 stainless steel balls (3 mm diameter). Extraction was made by adding 1.5 mL ethanol, vortexing for 2 min, followed by centrifugation for 5 min at 1500 rpm. The supernatant (100 μL) was diluted with 900 μL ethanol and the optical density read at 665 and 652 nm.

The concentration of chlorophyll *a* was calculated using Eq. 1.

$$Chlorophyll\ a\ (\mu g/mL) = (16.72 \times A_{665} - 9.16 \times A_{652}) \quad \text{(Eq.1)}$$

where A_{665}: absorbance at wavelengths of 665 nm; A_{652}: absorbance at wavelengths of 652 nm.

The percentage of total area (%TA) was calculated by the ratio between total area of fronds exposed to the mixture of drug and total area of fronds in control. The delta pH was calculated by subtracting the pH at the end of experiment with that at the beginning of the experiment. The same measurement for variables and end points evaluation was performed in *L. minor* and *S. auriculata*.

The percent inhibition of growth rate ($\%I_r$) was calculated with Eq. 2 (OECD 2006).

$$\%I_r = \frac{\left(\mu_{control} - \mu_{treatment\ group}\right)}{\mu_{control}} \times 100 \quad \text{(Eq.2)}$$

where μ is the average of specific growth rate calculate for each replicate using Eq. 3 (OECD 2006):

$$\mu_{i-j} = \frac{lnN_j - lnN_i}{t} \quad \text{(Eq.3)}$$

where μ_{i-j}: average specific growth rate; N_i: measurement variable in test at time *i* (beginning); N_j: measurement variable in test at time *j* (end); *t*: range time from *i* to *j*.

Individual drug test on the *L. minor* growth

Due to the greater sensitivity observed in *L. minor* for the mixed pharmaceutical growth trials compared to *S. auriculata*, further growth inhibition tests were performed to evaluate the influence of each pharmaceutical individually. The tests were performed in smaller vessels of 250 mL capacity in the same manner as described previously, with the concentration of each pharmaceutical set at 10, 200, and 500 μg L^{-1}. The same end points were evaluated as previously.

Statistics

The software Statistica (version 8.0, Stat Soft Inc., Tulsa, OK, USA) was used to perform all the statistical analyses. Basic statistic and normality tests were applied to the experimental data set.

Once the assumptions for the use of factorial ANOVA are (1) interval data of the dependent variable, (2) data normally distributed, (3) homoscedasticity, and (4) no multicollinearity, for the non-normally distributed variables (delta pH, %TA, and specific growth rate), the arcsin transformation was applied (arcsin(sqrt(variable)), as a variance stabilizer procedure.

Three-way analysis of variance (ANOVA) was used to identify the effects of drugs on macrophyte's growth (*L. minor* and *S. auriculata*) comparing control and treated groups with different concentrations and replication of the bioassay. The variability levels were 2 plant species (*L. minor* and *S. auriculata*), 3 concentrations of 8 drugs mix (0, 10, and 500 μg L^{-1}), and 3 replications of the 1-week bioassay. The growth inhibition trial was repeated thrice over 3 weeks (October 12 to 18, October 25 to November 1, and November 14 to 21 of 2017) with between 24 and 33 fronds (*n*) per tank for *Salvinia auriculata* and *Lemna minor*, respectively.

Later, for *L. minor*, two-way ANOVA was applied to assess the individual drug effect. Three end points and other related parameters were evaluated: frond number (FN), total area (TA) and chlorophyll *a*, percent inhibition of growth rate ($\%I_r$), specific growth rate (μ_{i-j}), and pH variation. The null hypothesis for the experiment is that the mean level of the macrophytes' parameter measured is the same irrespective of that determined under exposure to the drugs. The alternate hypothesis is that there is a difference on the mean growth of macrophytes between at least two groups (plant or drug concentration), presumably due to the presence of the drugs and/or by experimental setup due to the cultivation conditions.

The 0.05 level of significance was adopted.

Results

Three weeks replications

Table 2 presents results of total fronds number (FN), average specific growth rate (μ_{i-j}), percent inhibition of growth rate ($\%I_r$), total area of fronds (TA), percentage of total area related to the total area of control at day 7 (%TA), delta pH (difference between pH on day 7 and pH on day 0), and

Table 2 Response of macrophyte *L. minor* and *S. auriculata* following exposure to eight drugs at 10 and 500 μg L^{-1} as judged by frond development and chlorophyll *a* production

L. minor

	Week 1			Week 2			Week 3		
Concentration (μg L^{-1})	10	500	Control	10	500	Control	10	500	Control
Total no. fronds (day 0)	24	24	24	32	32	32	33	33	33
Total no. fronds (day 7)	41	23	64	182	47	249	98	43	122
SD	2	2	4	13	2	6	7	4	6
Specific GR μ_{i-j}	0.077	− 0.004	0.140	0.248	0.055	0.293	0.136	0.037	0.187
SD	0.011	0.014	0.013	0.013	0.009	0.005	0.035	0.014	0.010
$\%I_r$	44.655	103.200	-	15.389	81.328	-	27.284	79.991	-
Total area (cm^2)	0.659	0.466	0.917	8.911	2.422	14.222	2.763	2.425	4.166
SD	0.069	0.029	0.035	0.997	0.180	2.133	0.368	0.164	0.694
%TA	71.849	50.816	100.000	62.660	17.032	100.000	66.312	58.202	100.000
SD	7.524	3.182	3.801	7.011	1.269	14.997	8.833	3.934	16.658
delta pH	0.000	0.320	0.100	0.193	0.490	0.170	0.245	0.750	0.275
SD	0.014	0.029	0.010	0.017	0.029	0.030	0.035	0.016	0.035
Chlorophyll *a* (μg mL^{-1})	n.a.	n.a.	n.a.	3.487	0.658	5.932	0.308	0.640	2.077
SD	n.a.	n.a.	n.a.	0.307	0.067	0.972	0.045	0.136	0.334
Samples number (*n*)	3	3	2	3	3	2	2	3	2

S. auriculata

	Week 1			Week 2			Week 3		
Concentration (μg L^{-1})	10	500	Control	10	500	Control	10	500	Control
Total no. fronds (day 0)	24	24	24	24	24	24	16	16	16
Total no. fronds (day 7)	58	55	49	38	41	35	23	24	29
SD	3	4	4	2	4	0	1	2	4
Specific GR μ_{i-j}	0.125	0.118	0.100	0.067	0.076	0.054	0.054	0.058	0.081
SD	0.010	0.013	0.015	0.009	0.016	0.000	0.009	0.012	0.025
$\%I_r$	− 24.818	− 17.906	-	− 23.733	− 40.800	-	34.023	29.238	-
Total area (cm^2)	15.587	15.212	14.513	23.493	27.852	22.627	11.553	10.220	15.360
SD	1.832	1.518	1.978	1.664	4.481	2.415	1.389	1.054	1.063
%TA	107.403	104.814	100.000	103.826	123.094	100.000	75.214	66.534	100.000
SD	12.622	10.460	13.629	7.356	19.804	10.675	9.043	6.863	6.918
delta pH	− 0.047	− 0.333	− 0.140	− 0.250	− 0.557	− 0.165	− 0.183	− 0.827	− 0.225
SD	0.005	0.029	0.000	0.029	0.005	0.005	0.009	0.017	0.005
Chlorophyll *a* (μg mL^{-1})	n.a.	n.a.	n.a.	18.979	22.912	20.160	7.515	4.979	8.733
SD	n.a.	n.a.	n.a.	3.041	2.189	2.622	1.575	0.489	1.973
Samples number (*n*)	3	3	2	3	3	2	3	3	2

SD: standard deviation; (-) not calculated; n.a. not available.

chlorophyll *a* for *L. minor* and *S. auriculata* in growth inhibition test.

SD, standard deviation; (-) not calculated; *n.a.*, not available

Considering the inhibition growth rate ($\%I_r$) for concentration of 10 μg L^{-1}, a coefficient of variation of 50% was observed through results of 3 weeks, with an average of 30% of inhibition of *L. minor*. This variation can be associated with the low concentration tested. At concentration of 500 μg L^{-1}, the variation coefficient was less than 15% with an average of 88% of $\%I_r$. Comparing the total area of fronds treated with the mixture of drugs in concentrations of 10 and 500 μg L^{-1}, with the total area of fronds of the control, it is observed that there was a decrease in the total area of 67% and 43%, respectively.

Regarding the pH measurement from the beginning to the end of the test, it was observed that there was a small variation of 3.3%, 2.5%, and 9.5% of the pH (control, 10, and 500 μg

L^{-1}, respectively), indicating that the pH variation was correlated to the tested compounds concentration from day 0 to day 7. There was a considerable reduction in the concentration of chlorophyll *a* after exposure to the mixture of 8 drugs at the concentration of 500 μg L^{-1} in the test of weeks 2 and 3, which probably means that the high concentration can cause chlorosis to the aquatic plants.

The results obtained at the determined end points were statistically tested to verify whether these variations were significant or not. Table 3 presents the 3-way ANOVA results of the bioassay tests using the mixtures of 8 drugs at the two concentration levels and control. The replicate variability explained between 6 and 21% of variable's response, for %TA and for both TA and chlorophyll *a*, respectively.

Considering all variables presented in Table 3, there was significant difference between the replication test (R) effect isolated or combined with the plant (P) and concentration (C). Our assessment is that the 3-way ANOVA was able to discriminate the random variability from R test, and that variability was isolated from the other effects P and C. Once temperature and photo period were not controlled, such variability was considered normal. However, the ability of the statistical tests to separate random effects was mandatory under these circumstances. Thus, the R variability did not hinder the observation of other two studied factor effects. The three-way ANOVA was able to discriminate between the R variability and the variabilities attributed to the plant species (P) and to the 8 drug mix C levels.

Two macrophyte species response to the mix of eight drugs

Table 3 presents the three-way ANOVA result of the bioassay tests for *L. minor* and *S. auriculata* using the mixtures of 8 drugs at the two Cs tested and control. There was a significant difference observed between the growth of *L. minor* and *S. auriculata*.

Despite the large variability observed between the three Rs (Fig. 2) in the macrophyte's FN, it was possible to identify an effect of the pharmaceuticals on the *L. minor* that was not observed for *S. auriculata*. For *L. minor*, this was significant at both Cs (10 and 500 μg L^{-1}). Similar behavior was observed to the other growth variables (not graphically presented, but listed in Table 3).

Figure 3 presents the macrophytes growth responses, when R variability was removed (P vs C effects), for FN, TA, %TA, chlorophyll *a*, and specific GR. The R variability was removed in this case by presenting the least square mean (LS mean), that is, a calculated mean when the selected covariate is controlled or removed. The calculation is possible due to the orthogonality in the sum of variances. The values obtained by the *F*-test indicate that the mixture of drugs was able to cause significant difference in delta pH, total FN, TA, and on the average specific growth rate for *L. minor*. This effect was enhanced with increased C, whereas no influence of the drug mixture on the growth of *S. auriculata* was observed. For *L. minor*, growth suppression caused by exposure to 10 and 500 μg L^{-1} was approximately 30 and 70%, respectively.

Effect of single and mixed drugs on development of the macrophyte *L. minor*

The FN and chlorophyll *a* C were measured under single drug exposure and again under mixed drug exposure. Table 4 presents results of FN at day 7, μ_{i-j}, $\%I_r$, TA, %TA, pH on day 0, pH on day 7, and chlorophyll *a* for *L. minor* in growth inhibition test.

$\overline{A}$, average; *SD*, standard deviation; "-": not applicable

Through the results from growth test with drugs individually (Table 4), it can be observed that there was a stimulation on the growth rate for AMO, CAF, LOS, IBU, DIP, CBZ, and MIX in C of 10 μg L^{-1}, but the C of chlorophyll *a* was higher than control only for AMO, CAF, and LOS. The Cs of chlorophyll *a* for the other compounds remained almost the same as control or lower, even for TEN which had an inhibition on growth rate of 30%. At C of 500 μg L^{-1}, a stimulation in growth rate was observed for AMO, CAF, LOS, IBU, OME, and CBZ with an increase in chlorophyll *a* C. The exposure to higher C causes inhibition only for TEN (98.7%) and MIX (94.9%) with a decrease in chlorophyll *a* C, indicating that the treatment caused chlorosis to *L. minor*.

For most tests, the addition of pharmaceutical compounds raised the pH, approaching to pH 6. This increase in pH may have provided better conditions for plant growth, as described by Ekperusi et al. (2019), which recommend pH between 6 and 7.5 as ideal for growth of *L. minor* in laboratory.

The coefficient of variation among $\%I_r$ for drug mixture of Test 2 and Test 1 (Fig. 1), in C of 500 μg L^{-1}, was 13% and both experiments showed a growth inhibition of *L. minor*.

A two-way ANOVA was applied to determine if the variation determined by the simultaneous effect of individual drug treatment and replication observed is significant or not. Table 5 shows that at least one of single and mixed drugs treatments caused an effect statistically different from the control on the total FN and on the chlorophyll *a* C.

df, degree of freedom; *SS*, sums of square; *MS*, means square; *F* = (variability between groups/variability within groups); *p*, significance level; *Var%*, percentage of variance

Figure 4 presents the impact of the single and mixed drugs on the growth parameters when the batch variability is the controlled covariate.

Statistically significant reduction in the FN of *L. minor* was only observed with exposure to TEN and the mixture of all drugs. *L. minor* chlorophyll *a* C was more susceptible to change than the FN to the drug effect. Significant statistical

Table 3 Three-way analysis of variance experiment over on *L. minor* and *S. auriculata* growth, considering the plant (P)*replication test (R)*concentration effect (C)

delta pH						
Effect	*df*	SS	MS	*F*	*p*	Var%
P	1	0.007	0.007	7.4	**0.011**	0%
R	2	0.508	0.254	284.0	**0.000**	20%
C	2	1.681	0.841	940.5	**0.000**	66%
P*R	2	0.004	0.002	2.1	0.145	0%
P*C	2	0.006	0.003	3.3	**0.049**	0%
R*C	4	0.225	0.056	62.9	**0.000**	9%
P*R*C	4	0.011	0.003	3.1	**0.032**	0%
Error	30	0.027	0.001	-	-	1%
Transformed delta pH*						
Effect	*df*	SS	MS	*F*	*p*	Var%
P	1	0.018	0.018	12.8	**0.001**	1%
R	2	0.833	0.416	288.4	**0.000**	12%
C	2	2.273	1.137	787.2	**0.000**	33%
P*R	2	0.011	0.005	3.8	**0.035**	0%
P*C	2	0.008	0.004	2.8	0.076	0%
R*C	4	0.246	0.061	42.5	**0.000**	2%
P*R*C	4	0.028	0.007	4.9	**0.004**	0%
Error	30	0.018	0.018	12.8	**0.001**	1%
FN						
Effect	*df*	SS	MS	*F*	*p*	Var%
P	1	35,724	35,724	606.9	**0.000**	24%
R	2	23,952	11,976	203.5	**0.000**	16%
C	2	19,911	9955	169.1	**0.000**	14%
P*R	2	35,063	17,532	297.8	**0.000**	24%
P*C	2	22,548	11,274	191.5	**0.000**	15%
R*C	4	9824	2456	41.7	**0.000**	7%
P*R*C	4	10,746	2686	45.6	**0.000**	7%
Error	30	1766	59	-	-	1%
TA (cm^2)						
Effect	*df*	SS	MS	*F*	*p*	Var%
P	1	1850	1850	361.7	**0.000**	52%
R	2	757	378	74.0	**0.000**	21%
C	2	19	10	1.9	0.172	1%
P*R	2	84	42	8.2	**0.001**	2%
P*C	2	63	31	6.1	**0.006**	2%
R*C	4	12	3	0.6	0.671	0%
P*R*C	4	166	42	8.1	**0.000**	5%
Error	30	153	5	-	-	4%
%TA						
Effect	*df*	SS	MS	*F*	*p*	Var%
P	1	64,772.81	64,772.81	561.3	**0.000**	71%
R	2	5099.12	2549.56	22.1	**0.000**	6%
C	2	856.39	428.19	3.7	**0.036**	1%
P*R	2	3145.23	1572.62	13.6	**0.000**	3%
P*C	2	875.04	437.52	3.8	**0.034**	1%
R*C	4	553.55	138.39	1.2	0.332	1%
P*R*C	4	2737.82	684.46	5.9	**0.001**	3%
Error	30	3461.70	115.39	-	-	4%
Transformed %TA*						
	df	SS	MS	*F*	*p*	Var%
P	1	0.374	0.375	8625.4	**0.000**	97%
R	2	0.003	0.003	79.2	**0.000**	1%
C	2	0.001	0.001	3.9	**0.030**	0%
P*R	2	0.003	0.001	31.2	**0.000**	1%
P*C	2	0.001	0.001	15.8	**0.000**	0%
R*C	4	0.002	0.001	26.5	**0.000**	1%
P*R*C	4	0.001	0.001	1.8	0.150	0%
Error	30	0.002	0.001	8.9	**0.000**	1%
Chlorophyll *a*						
Effect	*df*	SS	MS	*F*	*p*	Var%
P	1	843	843	133.6	**0.000**	41%
R	1	434	434	68.7	**0.000**	21%
C	2	2	1	0.1	0.864	0%
P*R	1	214	214	33.9	**0.000**	10%
P*C	2	32	16	2.5	0.104	2%
R*C	2	6	3	0.5	0.631	0%
P*R*C	2	49	25	3.9	**0.037**	2%
Error	20	126	6	-	-	6%
Specific GR						
Effect	*df*	SS	MS	*F*	*p*	Var%
P	1	0.028	0.028	129.7	**0.000**	11%
R	1	0.016	0.008	36.1	**0.000**	3%
C	2	0.059	0.029	136.1	**0.000**	12%
P*R	1	0.058	0.029	134.6	**0.000**	12%

Table 3 (continued)

delta pH						
Effect	*df*	SS	MS	*F*	*p*	Var%
P*C	2	0.066	0.033	151.5	**0.000**	13%
R*C	2	0.006	0.001	6.7	**0.001**	1%
P*R*C	2	0.010	0.002	11.7	**0.000**	1%
Error	20	0.007	0.000			
Transformed specific GR*						
Effect	*df*	SS	MS	*F*	*p*	Var%
P	1	0.000	0.000	29.5	**0.000**	4%
R	1	0.000	0.000	17.0	**0.000**	2%
C	2	0.002	0.001	106.8	**0.000**	14%
P*R	1	0.002	0.001	91.3	**0.000**	12%
P*C	2	0.002	0.001	118.3	**0.000**	16%
R*C	2	0.000	0.000	1.4	0.246	0%
P*R*C	2	0.000	0.000	3.5	**0.018**	0%
Error	20	0.000	0.000	29.5	**0.000**	4%

df, degree of freedom; *SS*, sums of square; *MS*, means square; *F* = (variability between groups/variability within groups); *Var%*, percentage of variance; replication of test refers to the potential for between test variability. Values in bold present the effects and variables with significant statistical difference (*p*-value: $p < 0.05$). * arcsin transformation (arcsin(sqrt(variable))

differences were found on chlorophyll *a* content when CAF, LOS, IBU, OME, CBZ, and TEN were used, as well as under the mixed drug test. However, CAF, LOS, IBU, OME, and CBZ increased chlorophyll *a* content while TEN and the mixture of drugs suppressed the chlorophyll *a* C on *L. minor*. This potentially indicates that repeated exposure to those drugs could lead to excessive growth of this macrophyte and thus an imbalance in the environment.

Considering the single and mixed drugs test, some degree of effects interaction was observed (up to 30% of the variance), which was considered acceptable under experimental conditions. It is recognized that the *F*-test assesses the equality of variances under each effect condition (when $F_{calculated} >> F$ from the *F*-distribution, a significant statistical difference is observed between groups) and that a single test is performed to detect any of several possible differences. In this study, the alternative hypothesis was true, stating the difference between some of the single drugs, the drug mix from the control samples. However, in this case, in spite of the drug mix and TEN presented a similar response, both the statistical

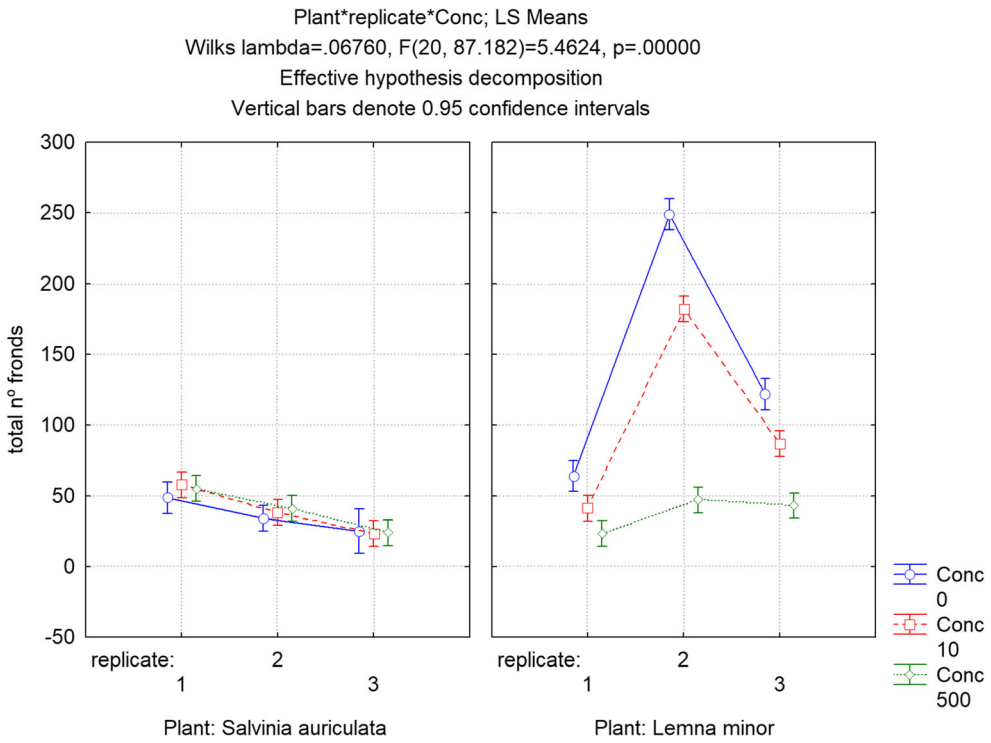

Fig. 2 LS means by P*R*C effects over the *Salvinia auriculata* and *Lemna minor* growth, with 8 drug mix. Bar errors correspond to 0.95 confidence interval: total no. fronds

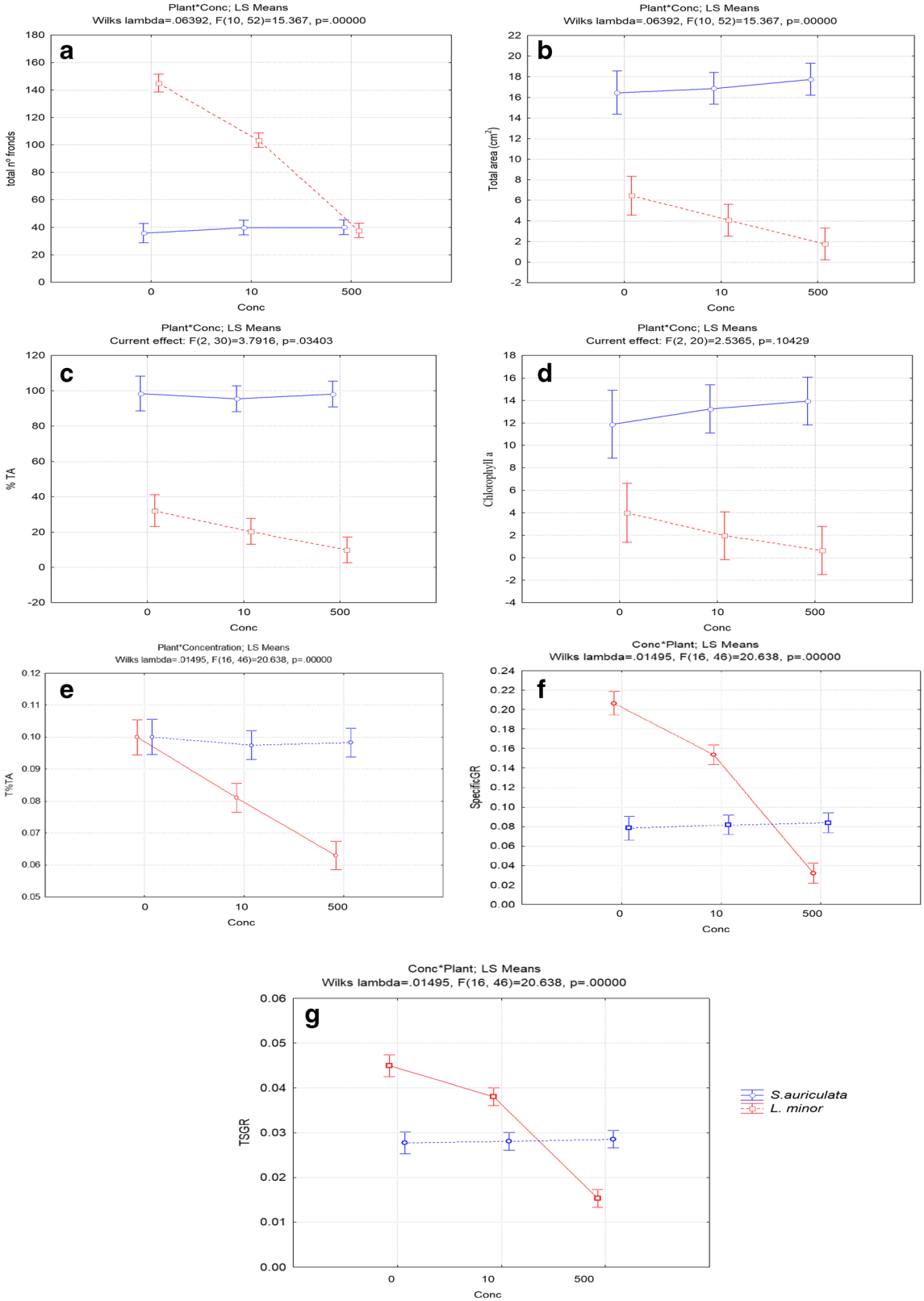

Fig. 3 LS means on *Salvinia auriculata* (blue) and *Lemna minor* (red) growth with replication test variability as controlled covariate. Bar errors correspond to 0.95 confidence interval: **a** total no. fronds; **b** total area (cm^2); **c** %TA; **d** chlorophyll *a*; **e** transformed %TA; **f** specific GR; **g** transformed specific GR

Table 4 Individual and mixed pharmaceuticals effect on growth and chlorophyll *a* production of *Lemna minor*

C 10 μg L^{-1}

		FN	μ_{i-j}	%I_r	TA (cm^2)	%TA	pH day 0	pH day 7	Chlorophyll *a* (μg mL^{-1})
Control	$\overline{A}$	39	0.136	-	2.007	100.000	5.54	5.60	0.728
	SD	1	0.004	-	0.119	5.914	0.03	0.02	0.018
AMO	$\overline{A}$	43	0.149	− 9.404	1.984	98.866	5.51	5.62	0.811
	SD	1	0.004	-	0.076	3.808	0.02	0.02	0.022
CAF	$\overline{A}$	52	0.177	− 29.393	2.576	128.375	5.49	5.72	1.035
	SD	2	0.006	-	0.031	1.539	0.01	0.01	0.008
LOS	$\overline{A}$	55	0.185	− 35.824	2.823	140.688	5.53	5.69	0.756
	SD	3	0.010	-	0.178	8.854	0.02	0.03	0.047
IBU	$\overline{A}$	51	0.174	− 27.345	2.616	130.375	5.59	5.73	0.703
	SD	2	0.007	-	0.210	10.482	0.01	0.01	0.048
DIP	$\overline{A}$	39	0.138	− 0.796	2.004	99.862	5.60	5.67	0.738
	SD	2	0.008	-	0.178	8.850	0.02	0.03	0.016
OME	$\overline{A}$	37	0.124	8.885	2.261	112.685	5.63	5.70	0.982
	SD	8	0.038	-	0.238	11.882	0.00	0.02	0.041
TEN	$\overline{A}$	30	0.095	30.633	1.447	72.106	5.67	5.66	0.714
	SD	6	0.035	-	0.131	6.529	0.01	0.01	0.072
CBZ	$\overline{A}$	56	0.188	− 37.818	2.591	129.142	5.70	5.70	0.597
	SD	2	0.007	-	0.137	6.832	0.01	0.03	0.082
MIX	$\overline{A}$	50	0.171	− 25.039	2.197	109.479	5.73	5.77	0.518
	SD	4	0.013	-	0.133	6.629	0.01	0.02	0.050

C 200 μg L^{-1}

		FN	μ_{i-j}	%I_r	pH day 0	pH day 7	Chlorophyll *a* (μg mL^{-1})
Control	$\overline{A}$	62	0.203	-	5.46	6.12	0.982
	SD	0	0.001	-	0.05	0.07	0.079
AMO	$\overline{A}$	54	0.177	12.841	5.48	6.17	0.809
	SD	13	0.046	-	0.02	0.02	0.161
CAF	$\overline{A}$	69	0.218	− 7.358	5.51	6.53	1.146
	SD	4	0.010	-	0.01	0.03	0.369
LOS	$\overline{A}$	55	0.184	9.435		6.13	1.237

Table 4 (continued)

C 10 μg L^{-1}									
	SD		7	0.024	-	5.54		0.06	0.180
IBU	$\overline{A}$		53	0.180	11.302	0.01		6.48	1.093
	SD		5	0.018	-	5.49		0.05	0.042
DIP	$\overline{A}$		51	0.173	14.866	0.02		6.08	0.926
	SD		5	0.017	-	5.53		0.02	0.122
OME	$\overline{A}$		56	0.188	7.511	0.01		6.47	1.124
	SD		6	0.019	-	5.57		0.04	0.103
TEN	$\overline{A}$		24	0.070	65.571	0.01		6.63	0.573
	SD		1	0.003	-	5.54		0.17	0.133
CBZ	$\overline{A}$		45	0.155	23.907	0.01		6.51	0.894
	SD		7	0.031	-	5.51		0.03	0.206
MIX	$\overline{A}$		24	0.064	68.368	0.01		6.67	0.430
	SD		2	0.018	-	5.56		0.11	0.015
C 500 μg L^{-1}						0.01			
		FN	μ_{i-j}	$\%I_r$	TA (cm^2)	%TA	pH day 0	pH day 7	Chlorophyll *a* (μg mL^{-1})
Control	$\overline{A}$	76	0.232	-	6.714	100.000	5.65	5.90	2.473
	SD	4	0.009	-	1.088	16.207	0.01	0.04	0.184
AMO	$\overline{A}$	94	0.262	− 12.782	9.056	134.884	5.66	5.89	3.238
	SD	6	0.011	-	0.984	14.655	0.01	0.05	0.181
CAF	$\overline{A}$	93	0.261	− 12.222	9.285	138.299	5.67	6.07	3.217
	SD	2	0.004	-	0.351	5.232	0.01	0.02	0.050
LOS	$\overline{A}$	92	0.258	− 11.206	9.517	141.753	5.67	5.99	3.495
	SD	6	0.012	-	1.016	15.130	0.02	0.09	0.327
IBU	$\overline{A}$	81	0.241	− 3.675	8.321	123.932	5.66	6.05	3.269
	SD	4	0.008	-	0.127	1.888	0.01	0.07	0.072
DIP	$\overline{A}$	73	0.225	3.200	6.925	103.150	5.66	6.05	2.810

Table 4 (continued)

C 10 μg L^{-1}										
	SD	9	0.024	-	1.476	21.978	0.02	0.28	0.264
OME	$\overline{A}$	85	0.246	− 5.764	8.859	131.950	5.66	6.14	3.270
	SD	14	0.031	-	1.146	17.071	0.02	0.18	0.210
TEN	$\overline{A}$	15	0.004	98.404	1.093	16.275	5.66	6.01	0.425
	SD	0	0.005	-	0.124	1.845	0.01	0.02	0.062
CBZ	$\overline{A}$	95	0.263	− 13.232	10.588	157.706	5.68	6.02	3.584
	SD	9	0.018	-	0.953	14.190	0.03	0.06	0.215
MIX	$\overline{A}$	16	0.012	94.802	1.376	20.493	-	6.33	0.525
	SD	1	0.013	-	0.278	4.139	-	0.09	0.044

$\overline{A}$:Average; SD: standard deviation; '-': not applicable.

test and the experimental procedure do not allow a clear identification of which drug was responsible by that difference. Some degree of synergism and antagonism in the drug mix could occur that the statistical test cannot isolate with the available data.

Discussion

The present study evaluated the effect of 8 drugs, individually and in mixture, on the growth of *L. minor* and *S. auriculata*. There is a gap of knowledge about influence of drugs on aquatic plants, mainly *S. auriculata*. Based on results obtained in this study, it seems to be an optimal relationship between drug C and frond growth, since some studies observed inhibition in frond growth of *L. minor* when exposed to the same drugs at higher Cs than those used here, which is the opposite response observed in this study.

Some studies about influence of IBU on growth inhibition contradict each other. Pomati et al. (2004) observed inhibition in the growth of *L. minor* caused by IBU in the range of 1 to 1000 μg L^{-1}, with a reduction of up to 25% in relation to the control for the 1000 μg L^{-1} C after 7 days of exposure, while Pietrini et al. (2015) evaluated the influence of IBU at 1 mg L^{-1} on the development of *L. gibba* during an 8-day laboratory experiment and observed about a 12% increase in the FN in relation to the control and no significant change in relation to chlorophyll *a*. The main difference between both studies can be explained with an observation made by Pomati et al. (2004): they reported that on the 2nd day of exposure, the presence of the drug stimulated the growth of *L. minor*, and after culture medium was renewed on the 5th day of exposure, an inhibition of plant growth was observed. In the Pomati et al. (2004) study, the authors' hypothesis was that IBU decomposes into metabolites which have properties of stimulating the growth of plants, in an optimal C of 10 μg L^{-1} since in higher Cs, similar results were not obtained.

In this study, *L. minor* showed growth stimulation at the C of 500 μg L^{-1} of IBU. Di Baccio et al. (2017) evaluated the effects of *Lemna gibba* exposure to IBU in Cs of 0.02, 0.2, and 1 mg L^{-1} and found no significant difference on the FN, chlorophyll *a*, or leaf necrosis compared to control. However, Nunes et al. (2014) compared the effects of the drug acetaminophen (paracetamol) on *L. gibba* and *L. minor*, with the effects being observed more intensely in *L. minor*, indicating that this species is more sensitive to adverse environmental conditions.

The results of growth inhibition test (Table 4) showed, for AMO, CAF, LOS, DIP, CBZ, and OME, an increase in FN at least for one C, which could be related to an increase in pH to close to 6. Ekperusi et al. (2019) described that a favorable environment to grow *L. minor* in laboratory consists of availability of nutrients (mainly N and P), adequate pH (between 6 and 7.5), exposure to light, and temperature. Additionally,

Table 5 Two-way analysis of variance of single and mixed drugs experiment on *Lemna minor* growth

Effect	*df*	Total frond number				
		SS	MS	*F*	*p*	Var%
R	2	11,844	5922	51.39	0.000	24%
Drug treatment	9	16,484	1832	15.90	0.000	33%
R * Drug treatment	18	14,738	819	7.11	0.000	30%
Error	60	6914	115			14%
Effect	*df*	Chlorophyll *a*				
		SS	MS	*F*	*p*	Var%
R	2	64.54	32.27	981.64	0.000	60%
Drug treatment	9	20.01	2.22	67.62	0.000	19%
R * Drug treatment	18	20.07	1.12	33.92	0.000	19%
Error	60	1.97	0.03			2%
Effect	*df*	Average specific GR				
		SS	MS	*F*	*p*	Var%
R	2	0.04	0.02	52.11	0.000	8%
Drug treatment	9	0.25	0.03	77.43	0.000	57%
R * Drug treatment	18	0.13	0.01	20.40	0.000	30%
Error	60	0.02	0.00			5%
Effect	*df*	Transformed specific GR				
		SS	MS	*F*	*p*	Var%
R	2	0.018	0.009	4.8	0.000	1%
Drug treatment	9	0.902	0.1	54.4	0.000	61%
R * Drug treatment	18	0.458	0.025	13.8	0.000	31%
Error	60	0.111	0.002			
Effect	*df*	delta pH				
		SS	MS	*F*	*p*	Var%
R	2	0.873	0.097	8.9	0.000	8%
Drug treatment	9	8.573	4.286	393.9	0.000	78%
R * Drug treatment	18	0.889	0.049	4.5	0.000	8%
Error	60	0.653	0.011			

df: degree of freedom; SS: sums of square; MS: means square; F = (Variability between groups/Variability within groups); p: significance level; Var%: Percentage of variance.

Wang et al. (2016) described the consumption of inorganic N by plants can be in the form of nitrate (NO_3^-) or ammonium (NH_4^+) which is more easily metabolized mainly for plants with a simple structure such as *L. minor*, being therefore called "NH_4^+ specialist." However, an excess of nutrient such as NH_4^+ can unbalance the aquatic environment causing toxic effects.

For AMO, growth stimulation could be a response of an increase in the availability of NH_4^+ and NO_3^- from photodegradation of N molecule in its structure. Pereira (2014) evaluated AMO removal processes for treatment and observed that when using the TiO_2 solar photocatalysis method in pure AMO solution, there was a transformation of 30% of the N of the drug molecule to NH_4^+. Klauson et al. (2010) and Elmolla and Chaudhuri (2010) described the degradation pathway of AMO molecule by photocatalytic oxidation into ammonium, nitrate, water, and carbon dioxide. Additionally, Low et al. (1991) and Elmolla and Chaudhuri (2010) reported that compounds with N in aliphatic chain, saturated ring, and aromatic ring can decompose into ammonium and nitrate, although compounds with N in aliphatic chain produce ammonium and nitrate faster and in higher C.

This transformation pathway should be investigated for CAF, LOS, DIP, CBZ, and OME since they also have N in their molecular composition.

TEN and the mixture of 8 drugs showed inhibition on growth of *L. minor* and significant statistical difference between the tested Cs and the control solutions for all end points evaluated. To the best of the authors' knowledge, none so far have evaluated the effect of tenivastatin, or even simvastatin (which has similar structure), on aquatic plant growth.

To evaluate the real risk of micropollutants in the environment, ecotoxicological tests on aquatic plants such as *L. minor* should be performed assessing different end points because effects of some substances can be more pronounced for other variables than the FN. This should include not only the observation of macro variations in FN, biomass production, and leaf area but also effects at biochemical and histochemical levels. Kummerova et al. (2016) evaluated the effect of diclofenac and paracetamol after 10 days of exposure in *L. minor*, where inhibition of plant growth was observed at 100 μg L^{-1} C. For Cs of 0.1 and 10 μg L^{-1}, there was no significant effect for growth, but there was however change in histochemical parameters such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Alterations at this level occur before indications of adverse physiological and growth responses, indicating the beginning of an unbalance that possibly was masked.

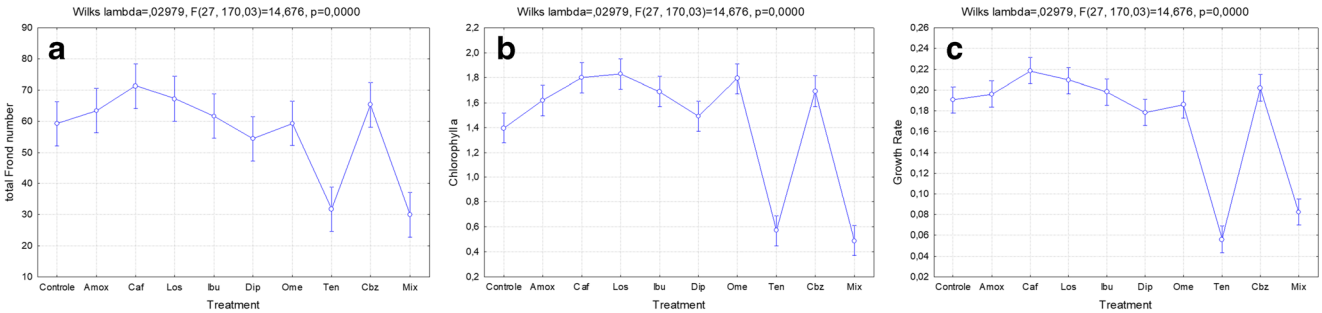

Fig. 4 LS mean by single and mixed drug effects with no R variability: **a** total frond number; **b** chlorophyll *a*; **c** average specific growth rate

For *S. auriculata*, most of studies in literature evaluate fronds and roots through microscope analysis, determining the influence of metals on the growth of these plants, like chlorosis, necrosis, epidermal and mesophyll cell collapse, and aerenchyma breakdown as end points, the latter 3 parameters are analyzed with the aid of a microscope. Studies evaluating the effects of drugs and mixture of drugs in *S. auriculata* are scarce.

Santos et al. (2020) analyzed the influence of a polluted river water samples in Pernambuco State (Brazil) on the growth of *S. auriculata*. It was observed no significant variation in the length of the submerged and floating leaves and ramets, chlorophyll *a*, the thickness of the cuticle, mesophyll, and central rib of the plant exposed to that sample in comparison with control. However, significant alterations at morphologic level were observed.

Conclusion

This study identified a remarkable difference in sensitivity between *L. minor* and *S. auriculata* that could be attributed to the individual morphological structure of these aquatic plants. Growth inhibition test showed that the mixture of eight drugs can affect the growth of *L. minor*, even at Cs of 10 μg L^{-1}. On the other hand, *S. auriculata* showed no growth change for the end points evaluated after exposure to a mixture of eight drugs. That persisted even when the mixed drug C was raised to 500 μg L^{-1}.

Examining the effect of individual drugs on *L. minor*, tenivastatin decreased the plant growth in 98.7% with the 500 μg L^{-1} C and in 65.5% with 200 μg L^{-1} C and even at environmentally relevant Cs (10 μg L^{-1}), this drug suppressed growth by 30.6%, representing a danger to aquatic life.

The drugs which stimulated growth can also offer a risk to aquatic ecosystem: many aquatic plants can block the infiltration of sunlight in the deeper layers of the water column and inhibit the production of submerged plants and algae, in addition to causing problems in navigation channels and clogging of hydroelectric turbines or catchment stations of water (Ekperusi et al. 2019). While individual drug evaluations may provide evidence of the potential risk of a particular compound and thus assist agencies responsible for the regulation of compounds that pose some biota risk, understanding the threat posed by mixtures is the key to comprehend the impact of compounds of emerging concern on the functions of complex natural and anthropic ecosystems. The work outlined here indicated that *L. minor* is a suitable macrophyte for use in pollutants screening.

More specific and complex parameters, such as ultrastructure, anatomy of leaf, histochemical, biochemical, and degradation pathway assessments, are required to evaluate the full sub-lethal effects of the drugs.

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Authors' contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by JIO, CH, JR, and KH. The statistical analysis was performed by LRM. The first draft of the manuscript was written by JIO and LRM, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article. The raw data are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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