Ecología Austral 31:053-064 April 2021
Argentine Association of Ecology
https://doi.org/10.25260/EA.21.31.1.0.1146

Effects of heavy metals, glyphosate and their binary mixtures on the growth of green algae.

COUSYAUZA AFIOUE DI C�isyofauo[1] ; ÁUGE�A B. Jvá�ez[2,3] ; JVAU Mo�eyyou[1] � Auaнí Magda�euo[1,]✉

[1]University of Buenos Aires, School of Pharmacy and Biochemistry, Chair of Public Health and Environmental Hygiene.
[2]University of Buenos Aires, Faculty of Exact and Natural Sciences, Department of Biodiversity and Experimental Biology. [3]CONICET-University of Buenos Aires, Institute of Biodiversity and Experimental and Applied Biology (IBBEA).

SUMMARY. Rivers and streams in rural areas of the Province of Buenos Aires contain variable concentrations of heavy metals and glyphosate. In the present study, the toxicity of the metals Cu, Pb and Zn, the herbicide glyphosate (active product and formulation ATANOR®) and their binary mixtures, was evaluated in two species of green algae (a standard [*Raphidocelis subcapitata*] and a native strain of *Scenedesmus acutus* isolated from Arroyo Burgos, Buenos Aires). The bioassays were carried out in a concentration range between
0.5 mg/L and 20 mg/L, and after 7 days of incubation the algal density was estimated. To obtain the effective concentrations inhibiting 10, 20 and 50% of the growth (EC10, EC20 and EC50) of each individual substance and of the mixtures by means of a nonlinear adjustment, the percentage of growth inhibition (%I) was modeled as a function of each concentration. According to the EC50 obtained, the toxicity of Cu and Zn was higher in *R. subcapitata* (7.47±2.14 and 6.51±2.26 mg/L, respectively) than in *S. acutus* (10.90±3.75 and >20 mg/L). Pb and glyphosate were not toxic to either strain. ATANOR glyphosate® was toxic only to *R. subcapitata* (EC50=12.00±3.10 mg/L). According to the EC10 and EC20 values of individual substances and binary mixtures, the *S. acutus* strain was more sensitive. Taking into account the toxic units (TUs) obtained, it was evident that the mixtures Cu+Zn, Cu+ATANOR® glyphosate and Zn+ATANOR® glyphosate showed antagonistic effects on *R. subcapitata* (according to TUs values obtained from EC20 and EC50), while the mixtures Cu+Pb and Cu+Zn had synergistic and antagonistic effects, respectively, on *S. acutus* (according to TUs values obtained from EC20). This study highlights the importance of conducting bioassays with toxic substances using native algal strains that allow us to infer potential effects on native communities and trophic webs.

[Keywords: copper, lead, zinc, toxicity].

ABSTRACT. Effects of heavy metals, glyphosate and their binary combinations on the growth of green algae. Rivers and streams of rural areas of the Buenos Aires Province contain variable concentrations of heavy metals and glyphosate. In this study, the toxicity of the metals Cu, Pb and Zn, the herbicide glyphosate (active product and ATANOR® formulation) and their binary combinations, was assessed on two green algae species (the standard species *Raphidocelis subcapitata* and a native strain of *Scenedesmus acutus* isolated from Burgos Stream [Buenos Aires]). The bioassays were carried out applying a concentration range of 0.5 mg/L - 20 mg/L, in incubations lasting 7 days after which the algal density was estimated. To obtain the effective concentrations inhibiting 10, 20 and 50% of the growth (EC10, EC20 and EC50) of individual substances and their combinations, the percentage of growth inhibition (%I) was modeled as a function of each concentration by a nonlinear fit. According to the EC50 obtained for each species, the toxicity of Cu and Zn was higher in *R. subcapitata* (7.47±2.14 and 6.51±2.26 mg/L, respectively) than in *S. acutus* (10.90±3.75 and> 20 mg/L), while Pb and glyphosate active compound were not toxic for neither of the two strains. ATANOR® glyphosate was toxic only to *R. subcapitata* (EC50=12.00±3.10 mg/L). According to the EC10 and EC20 values of the individual substances and the binary combinations, *S. acutus* showed higher sensitivity than *R. subcapitata*. From the analysis of toxic units (TU), the combinations Cu+Zn, Cu+glyphosate ATANOR® and Zn+glyphosate ATANOR® showed antagonistic effects on *R. subcapitata* (according to the TU values obtained from EC20 and EC50), while the Cu+Pb and Cu+Zn combinations showed synergistic and antagonistic effects, respectively, on *S. acutus* (according to the TU values obtained from the EC20). These results highlight the importance of conducting bioassays of toxic substances using native algal strains which allow inferring potential effects in native communities and food webs.

[Keywords: copper, lead, zinc, toxicity].

Associate Editor: María Diéguez

✉ amagda65@yahoo.com.ar

Received: May 12, 2020
Accepted: October 5, 2020

Iuy�odvccióu

Industrial and agricultural activities generate polluting substances that are distributed in different environments and affect the health of ecosystems and humans (Jorgensen et al. 1997). In Argentina, agriculture represents an important source of primary and secondary production, for which 31 million hectares are used. Transgenic crops (soybean, corn and cotton) cover three quarters of the cultivated land where herbicides, especially glyphosate, are used to control weeds during cultivation and fallow periods (Sarandón 2013). According to Aparicio et al. (2013), glyphosate adsorbed to soil particles can move through runoff processes into watercourses where it can accumulate in sediments and then desorb and pass into the liquid phase.

Glyphosate (N-phosphonomethylglycine) is a non-selective post-emergent herbicide whose main mechanism of action is to inhibit the enzyme 5-enolpyruvyl-shiquimate-3-phosphate synthetase, responsible for the formation of the aromatic amino acids phenylalanine, tyrosine and tryptophan in plants (Duke 1988). The impact of glyphosate in its different formulations on 'non-target' organisms has been the subject of extensive research over the last decade. Evidence of physiological effects on green algae, such as oxidative stress, reduction of superoxydodimutase and catalase activity, as well as growth inhibition, was reported (Romero et al. 2011). On the other hand, there is evidence that glyphosate and its formulations Roundup® and Atanor® affect the structure and function of phytoplankton and periphyton and, therefore, water quality and freshwater ecosystems (Pérez et al. 2007; Vera et al. 2010; Pizarro et al. 2015; González et al. 2019). It should be noted that glyphosate concentrations between 13 and 700 µg/ L were determined in surface waters of rivers and streams in the province of Buenos Aires (Peruzzo et al. 2008; Bollani et al. 2019).

Likewise, concentrations of heavy metals were detected in agricultural areas probably from agrochemicals, phosphate fertilizers and organic fertilizers (Fergusson 1990; Alloway 1995; Pignata et al. 2002), from wastewater used for irrigation, and from atmospheric depositions (Pignata et al. 2002; Kim et al. 2015). Elevated concentrations of heavy metals in the aquatic environment can lead to effects adverse effects on algae, such as reduced chlorophyll biosynthesis, inhibition of photosynthesis and growth (Omar 2002; Küpper et al. 2002).

Laboratory bioassays allow the bioavailability and interactive toxic effects of substances on aquatic organisms to be analyzed (Nowell et al. 2014). Photosynthetic algae in particular are selected models in ecotoxicological assays because they are fast-growing and can be grown in the laboratory using synthetic media that allow them to be maintained under optimal physiological conditions. In terms of their ecological importance, algae are the main primary producers of phytoplankton and the basis of aquatic food chains. The standard species proposed as a reference organism by several environmental agencies is the green alga *Pseudokircheriella subcapitata* (USEPA 2002; Environmental Canada 2007; ISO 2009). Currently, the valid taxonomic identity for this species is *Raphidocelis subcapitata*, based on genomic research by Suzuki et al. (2018). This species is sensitive to numerous toxicants, such as heavy metals and pesticides (Blinova 2004; Guéguen et al. 2004; Kahru et al. 2005; Magdaleno et al. 2015). However, the effects of contaminants on a standard species are not necessarily the same as those occurring in strains of species isolated from water bodies in an area of interest. Indigenous strains can provide relevant information that allows inferring the effects of contaminants in their environment (Magdaleno et al. 2014; Carusso et al. 2018).

On the other hand, in natural environments, living organisms are simultaneously exposed to a complex mixture of compounds that may interact additively, antagonistically or synergistically. Thus, the inclusion of the analysis of the effects of mixtures of two or more contaminants on living organisms is necessary to more accurately assess the risks of exposure in the natural environment. Recent studies detected the presence of elevated concentrations of heavy metals copper (Cu), lead (Pb) and zinc (Zn) and glyphosate in waters of Arroyo Burgos, a water body located in an agricultural-livestock area in the province of Buenos Aires (Bollani et al. 2019; Magdaleno et al. 2018). In this framework, this research

The objective of this study is to evaluate the effects of the metals Cu, Pb and Zn, and of glyphosate (active ingredient and formulation ATANOR®) and its binary mixtures on the growth of two species of green algae: a standard strain (*Raphidocelis subcapitata*) and a native strain of the species *Scenedesmus acutus* isolated from the Burgos stream.

Maye�ia�es �es � Méyodos

The strains of the two green algal species, *R. supcapitata* (Korshikov) and *S. acutus* (Meyen) (Chlorococcales, Chlorophyta) were obtained from the Culture Collection of the Laboratory of Phycology, Department of Biodiversity and Experimental Biology, Faculty of Exact and Natural Sciences, University of Buenos Aires. The standard species strain originally came from the Collection of Cultures of Algae and Protozoa (CCAP) with the identification 278/4. The autochthonous strain of the species *S. acutus* was isolated from Burgos stream (San Pedro, Province of Buenos Aires) and cultured axenically with the identification BAFC CA 14.

Stock solutions of 1 g/L of $Cu^{2+}/CuSO_4$, $Pb^{2+}/Pb(NO_3)_2$, $Zn^{2+}/ZnSO_4$, glyphosate (isopropylamine salt of N-phosphonomethyl glycine) and the formulation ATANOR® , containing 48% (w/v) glyphosate. From these solutions, different dilutions were made in Bold Basal Medium (BBM) for algal growth (Archibald and Bold 1970), in order to obtain the following test concentrations in mg/L: 0.5, 1, 2.5, 5, 7.5, 10 and 20. Heavy metal concentrations were measured by atomic absorption spectrophotometry and concentrations of glyphosate (active ingredient) and ATANOR glyphosate® were measured by high performance liquid chromatography (HPLC). According to these determinations, there was close to 95% similarity between the nominal and actual concentrations so that the former were considered throughout the work.

To perform the bioassays, 96-well plates were used, according to the protocol standardized by Environmental Canada (2007), using a final volume of 200 µL in each well (20 µL of inoculum+180 µL of toxicant or mixture). A modification of the protocol was the inclusion of BBM medium instead of the one proposed by the Canadian agency, because BBM contains the same nutrients, but in greater concentration, a condition more similar to eutrophicated water bodies in the Province of Buenos Aires. The initial inoculum was $1x10^5$ cells/mL. Each test was performed in quadruplicate. The control was performed with a total of 8 replicates with BBM medium, with no toxicant added. Binary mixtures were performed by incorporating equal parts of each substance (1:1) in order to obtain the same final concentrations as for individual substances. The plates were placed in a thermostated orbital shaker (22 °C), at 80 rpm speed and with continuous light for 7 days. The duration of the assay was set at 7 days since maximum population growth (up to $1x10^7$ cells/mL) of the algae in the controls was observed in that period under the mentioned conditions. At the end of the assay, algal density was estimated by absorbance readings at a wavelength of 620 nm, using a plate-reading spectrophotometer. This estimation was performed based on a standard calibration curve for each algal species by fitting the absorbance and cell concentration data with a first-order linear model (R^2 >0.95 [Carusso et al. 2018]). The assay was repeated between 5 and 7 times for each treatment and each algal strain.

A matrix was assembled from the absorbance data and descriptive statistics, one-factor ANOVA and *a posteriori* contrasts were applied using Tukey's test to evaluate significant differences between the absorbance of each treatment and the control. The statistical program R was used. For each concentration of toxicant, the percentages of inhibition (%I) with respect to the control were obtained, according to:

$$\%I = 100 \times [C - T] / C$$

where %I is the percentage inhibition of algal growth, C is the mean absorbance of the controls and T is the mean absorbance in each treatment. Different models were tested in order to select the best fit to explain the observed data. A nonlinear model was selected and graphs were made using GraphPadPrism software. The nonlinear function used is defined as the following sigmoid equation:

$$\%I = 100 \times (a^{HS} / 1 + X)^{HS}$$

where X is the concentration of the toxicant or mixture in mg/L, a corresponds to the mean value between 0% and 100% growth inhibition.

and HS is the slope describing the steepness of the curve. The fit was performed using the least squares method. From the fit parameters, the effective concentrations of each compound or mixture that produce 10, 20 and 50 percent growth inhibition (EC10, EC20 and EC50, respectively) were estimated.

To evaluate whether the combined effect of the two substances in each mixture was additive, synergistic or antagonistic, the concept of toxic unit (TU) was used, according to Broderius et al. (2005), which assumes that each component substance in the mixture has the same toxic mode of action. The joint effect is described as follows: if UT=1±0.2, the toxicity of the mixture is additive (concentration addition), if UT<0.8 represents potential synergism (more than additive), and if UT>1.2 indicates potential antagonism (less than additive) (Gonzalez-Pleiter et al. 2013). The following equation was used to calculate the UT of the mixtures:

UTmix = UTa + UTb

where, UTa is the toxic unit of component a of the mixture and UTb, the toxic unit of component b. The TUs of each component (a and b) were calculated according to the following equation:

UT = Ci / CE50

where, Ci is the concentration of component i (a or b) that produces a 50% inhibition. when both components are present in the mixture (the EC50 of the mixture), and EC50, the inhibitory concentration 50 of component i (a or b).

Resv�yados

Of the three metals tested, Cu and Zn were toxic to *R. subcapitata,* Cu was toxic to *S. acutus,* while Pb did not affect either species (Table 1). As for glyphosate, the ATANOR formulation® was toxic to the standard species, while the active ingredient was not toxic to the species studied. Considering the EC50 for Cu and Zn, *R. subcapitata* presented lower values than *S. acutus*, which would indicate that the standard species is more sensitive to these metals. However, as it appears from comparing the EC10 and EC20, the sensitivity of the native species was higher at low concentrations of the substances tested (Table 1). In both species, a dose-response effect was observed for Cu and Zn, while in *R. subcapitata* progressive increases in Pb concentrations did not inhibit its growth (Figure 1). A good fit to the nonlinear function was not obtained in *S. acutus* for Pb (R^2 =0.3); %I were less than 50% with an associated slope close to 0 (HS=-0.38±0.15), indicating a very low nonlinear relationship between %I and Pb concentrations (Table 2). ATANOR glyphosate® significantly inhibited *R. subcapitata* ($P<0.05$), while exposure to the active substance

Table 1. Effective concentrations 10, 20 and 50 (EC10, EC20 and EC50) of each individual substance obtained for the *Raphidocelis subcapitata* species strain and the *Scenedesmus acutus* species strain. The concentrations of the mixtures (1: 1) are expressed in mg/L.

Effective concentrations 10, 20 and 50 (EC10, EC20 and EC50) of each individual substance obtained for the strain of *Raphidocelis subcapitata* and the strain of *Scenedesmus acutus*. The concentrations of the mixtures (1:1) are expressed in mg/L.

Toxic	CE10	CE20	CE50
R. subcapitata			
Cu	0.83±0.98	3.09±2.67	7.47±2.14
Pb* Pb* Pb* Pb* Pb* Pb* Pb* Pb* Pb	-	-	-
Zn	0.05±0.04	0.12±0.12	6.51±2.26
Glyphosate (active ingredient)* Glyphosate (active ingredient)* Glyphosate (active ingredient)	-	-	-
ATANOR® glyphosate	0.11±0.06	0.24±0.08	12.08±3.15
S. acutus			
Cu	0.17±0.13	0.29±0.14	10.92±3.76
Pb	0.04±0.04	0.11±0.11	> 20
Zn	0.05±0.05	0.09±0.09	> 20
Glyphosate (active ingredient)* Glyphosate (active ingredient)* Glyphosate (active ingredient)	-	-	-

ATANOR®* glyphosate	-	

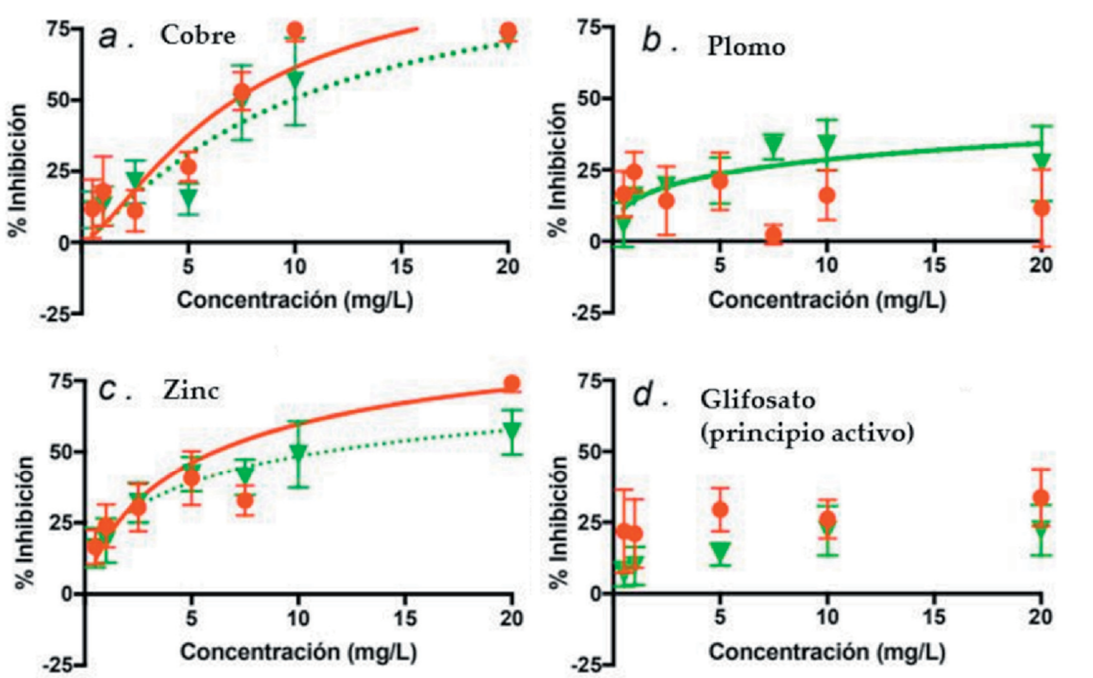
*No significant differences ($P<0.05$) were observed between the lowest concentration (0.5 mg/L) and the rest of the concentrations tested.

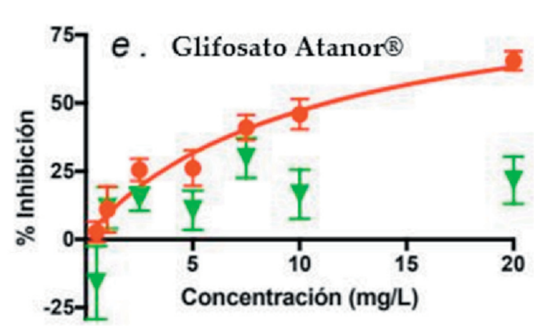
Values of the slope of the sigmoid curve (HS), of the value between 0% and 100% growth inhibition a, obtained for the respective fits of the %I data to the sigmoidal model, and their respective coefficients of determination (R^2) for each algal species and substance. The HS and a±ES estimators are shown.

Values of the sigmoid curve slope (HS), value between 0% and 100% of the inhibition growth a obtained for the respective adjustments of the %I data to the sigmoidal model, and their respective coefficients of determination (R^2) for each species and substance. HS estimators and a±SE are displayed.

Substance	HS	a	R2
R. subcapitata			
Cu	-1.40±1.40	7.17±0.31	0.85
Pb	NA	NA	NA
Zn	0.79±1.04	6.11±1.05	0.65
Glyphosate (active ingredient)	NA	NA	NA
ATANOR® glyphosate	-0.94±0.14	11.26±1.42	0.75
S. acutus			
Cu	-1.9±1.55	0.79±0.27	0.75
Pb	-0.38±0.15	110±50.4	0.3
Zn	-0.54±3.14	11.26±3.41	0.54
Glyphosate (active ingredient)	NA	NA	NA
ATANOR® glyphosate	NA	NA	NA

NA: did not fit the model

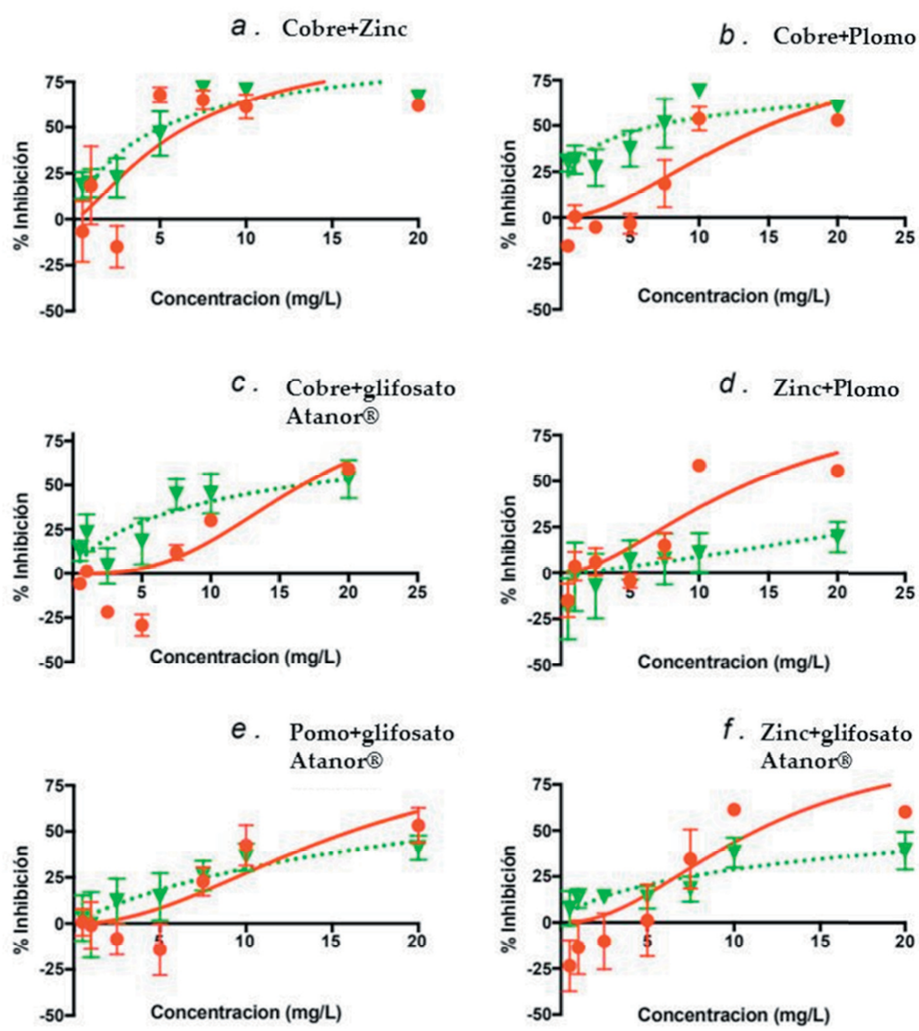




Population growth inhibition curves of *Raphidocelis subcapitata (*full line and red circle) and *Scenedesmus acutus* (dotted line and green triangle) at increasing concentrations of the individual substances.
(a) copper, (b) lead, (c) zinc, (d) glyphosate (active ingredient) and
e) glyphosate formulation ATANOR® , with fit to the sigmoidal model. The error bars represent the 95% CI.

Population growth inhibition curves of *Raphidocelis subcapitata* (solid line and circle in red) and *Scenedesmus acutus* (dotted line and triangle in green) at increasing concentrations of the individual substances a) copper, b) lead, c) zinc, d) glyphosate (active ingredient) and e) glyphosate ATANOR® formulation, with fit to the glyphosate did not significantly affect the growth of algal species ($P>0.05$).

To evaluate the effects of the binary mixtures on algal growth, the active ingredient glyphosate was excluded, since it was not inhibitory in any of the two species. In the three binary mixtures with Cu, growth stimulation was observed at low concentrations (0.5 and 1 mg/L) in *R. subcapitata* (Figure 2). At low concentrations of the mixtures, a higher toxicity was observed on *S. acutus* than in *R. subcapitata*, except for the Zn+Pb mixture. This trend was contrasting at higher concentrations, with the exception of the Cu+Pb mixture, in which toxicity was higher than in the native species at all concentrations, being equal at the highest concentration (20 mg/L) in both species (Figure 2). In all mixtures, lower EC10 and EC20 values were observed in *S. acutus* than in *R. subcapitata*, while EC50 values were similar in both species (Table 3). *S. acutus* did not show a dose-response effect for the Pb+Zn mixture in the range of concentrations and test conditions used for *S. acutus* reflecting the lack of convergence to the sigmoid curve model fit. In the three mixtures in which one of the components was ATANOR glyphosate®,



Population growth inhibition curves of *Raphidocelis subcapitata (*full line and circle in red) and *Scenedesmus acutus* (dotted line and triangle in green) at increasing concentrations of the binary mixtures. (a) Cu+Zn; (b) Cu+Pb; (c) Cu+glyphosate ATANOR® ; (d) Zn+Pb; (e) Pb+glyphosate ATANOR® ; (f) Zn+glyphosate ATANOR® , with fit to the sigmoidal model. The error bars represent the 95% CI.

Population growth inhibition curves of *Raphidocelis subcapitata* (solid line and circle in red) and *Scenedesmus acutus* (dotted line and triangle in green) at increasing concentrations of binary mixtures: a) Cu+Zn; b) Cu+Pb; c) Cu+glyphosate ATANOR®; d) Zn+Pb; e) Pb+glyphosate ATANOR®; f) Zn+glyphosate ATANOR®, with adjustment to the sigmoidal model. The error bars represent the 95% CI.

Table 3. Effective concentrations 10, 20 and 50 (EC10, EC20 and EC50) of the binary mixtures obtained for the algal species *R. subcapitata* and *S. acutus*. Concentrations of the mixtures (1:1) are expressed in mg/L.

Table 3. Effective concentrations 10, 20 and 50 (EC10, EC20 and EC50) of the binary mixtures obtained for the algal species *R. subcapitata* and *S. acutus*. The concentrations of the mixtures (1:1) are expressed in mg/L.

Mix	CE10	CE20	CE50
R. subcapitata			
Cu+Pb*	0.35±0.14	0.55±0.11	11.84±0.50
Cu+Zn*	0.17±0.14	0.25±0.13	8.23±4.10
Cu+Glyphosate ATANOR® * *	0.58±0.13	0.79±0.07	17.3±3.81
Cu+Glyphosate ATANOR			
Pb+Zn	0.32±0.16	0.5±0.14	15.99±5.47
Pb+Glyphosate ATANOR®	0.41±0.25	0.55±0.18	13.06±5.24
Zn+Glyphosate ATANOR®	0.39±0.18	0.59±0.13	19.70±8.08
S. acutus			
Cu+Pb	0.02±0.02	0.04±0.04	11.25±7.57
Cu+Zn	0.06±0.04	0.12±0.06	5.50±1.95
Cu+Glyphosate ATANOR®	0.12±0.12	0.21±0.19	>20
Pb+Zn	-	-	-
Pb+Glyphosate ATANOR®	0.13±0.12	0.25±0.23	>20
Zn+Glyphosate ATANOR®	0.18±0.18	0.31±0.25	>20

*Growth stimulation was observed at 0.5 and 1 mg/L concentrations.

Values of the slope of the sigmoid curve (HS), of the value between 0% and 100% growth inhibition a, obtained for the respective fits of the %I data to the sigmoidal model, and coefficients of determination (R^2) for each algal species and binary mixture. HS and a±ES estimators are shown.

Table 4. Values of the sigmoid curve slope (HS), value between 0% and 100% of the growth inhibition a obtained for the respective adjustments of the %I data to the sigmoidal model, and coefficients of determination (R^2) for each algal species and binary mixture. HS estimators and a±SE are displayed.

Binary blending	HS	a	$R2$
R. subcapitata			
Cu+Pb	-1.87±0.48	14.94±2.11	0.65
Cu+Zn	-1.38±0.50	6.55±1.46	0.52
Cu+Glyphosate ATANOR®	-2.93±0.77	16.62±1.63	0.66
Pb+Zn	-1.77±0.49	13.46±2.02	0.69
Pb+Glyphosate ATANOR®	-2.05±0.68	16.08±2.76	0.48
Zn+Glyphosate ATANOR®	-2.09±0.84	11.32±2.11	0.47
S. acutus			
Cu+Pb	-0.46±0.13	6.87±2.23	0.37
Cu+Zn	-0.87±0.17	5.12±0.88	0.68
Cu+Glyphosate ATANOR®	-0.73±0.24	16.57±6.13	0.35
Pb+Zn	NA	NA	NA
Pb+Glyphosate ATANOR®	-0.93±0.38	25.03±11.82	0.33
Zn+Glyphosate ATANOR®	-0.65±0.21	39.65±22.49	0.35

NA: did not fit the model

EC50 values were greater than 20 mg/L for *S. acutus*. In these cases a concentration value could not be obtained, since no inhibition of algal growth occurred in the concentration range used.

From an overall perspective, the autochthonous strain of *S. acutus* presented notably weaker adjustments than the standard strain of *R. subcapitata* (Table 4). No adjustment explained more than 40% of the variance of the

%I with the exception of the Cu+Zn mixture, which presented the best fit of that series (R^2 =0.68). The values of the slopes estimated for

S. acutus were lower (Table 4).

To evaluate possible synergistic or antagonistic effects of the mixtures on algae, the toxic units UT20 and UT50 were calculated from the respective EC20 and EC50 of the binary mixtures divided by the EC20 and EC50 of the individual toxicants (Table 5). The values

Toxic Units (TU) obtained for the binary mixtures from the EC20 and EC50 of the individual substances and the binary mixtures for the species *R. subcapitata* and *S. acutus*.

Table 5. Toxic Units (TU) obtained for the binary mixtures from the EC20 and EC50 of the individual substances and binary mixtures for the algal species *R. subcapitata* and *S. acutus*.

Mix	CE20	CE50	UT20	UT50
R. subcapitata				
Cu	3.09	7.47		
Zn	2.67	6.51		
ATANOR Glyphosate®	0.24	12.08		
Cu+Zn	0.25	8.23	2.16	2.35
Cu+Glyphosate ATANOR®	0.79	17.3	3.54	3.75
Zn+Glyphosate ATANOR®	0.59	19.70	6.87	3.08
S. acutus				
Cu	0.29	10.92		
Pb	0.11	-		
Zn	0.09	-		
Cu+Pb	0.04	-	0.47	-
Cu+Zn	0.12	-	1.74	-

of UT20 and UT50 as determined in the case of *R. subcapitata* showed antagonistic effects in the three mixtures obtaining EC20 and EC50 values for both mixtures and individual substances, i.e. Cu+Zn, Cu+glyphosate ATANOR® and Zn+glyphosate ATANOR® . The UT values were higher when the glyphosate formulation was present in the mixture. In the case of *S. acutus,* UT50 could not be calculated due to the absence of EC50 values for the individual substances (with the exception of Cu), and therefore only UT20 was calculated. In this case ATANOR glyphosate® did not show a dose-response effect on algal growth when confronted individually. Only two mixtures allowed the approach from the TUs, the Cu+Pb mixture, which presented a value of TU<1 and the Cu+Zn mixture, with a value of TU>1 (Table 5). This indicates that the former produced a synergistic effect on the native species and the latter an antagonistic effect.

DISCUSSION

The EC50 obtained for Cu and Zn metals in *R. subcapitata* (7.47 and 6.51 mg/L, respectively) were considerably higher than those reported in the literature (between 0.01 and 0.13 mg/L for Cu and between 0.05 and 0.31 mg/L for Zn) for that species (Guéguenetal. 2004; Geisetal. 2000; Magdaleno et al. 2014). On the other hand, these authors reported EC50 values for Pb ranging from 0.29 and 2.7 mg/L, while in the present work no dose-response effect was obtained over the range of concentrations 0.5 to 20 mg/L. One of the advantages of using of algal bioassays to assess surface water quality is that they provide information on both growth inhibition produced by the presence of toxicants and their stimulation due to nutrient concentration (Magdaleno et al. 2018). In this work, bioassays were carried out using a nutrient-rich culture medium (BBM), which contains higher concentrations of N and P than the media used in other works. The growth stimulation due to the high nutrient concentrations would partially counteract the inhibitory effect of metals in solution, reflected in the high EC50 values obtained. It should be noted that the use of a nutrient-rich culture medium is more satisfactory to establish conditions similar to those of the rivers and streams of the Province of Buenos Aires, which are generally eutrophic. For example, in the Burgos stream (locality of San Pedro), from where the *S. acutus* species strain was isolated, high concentrations of N and P (14.2 and 14.9 mg/L, respectively) were found (Bollani et al. 2019).

On the other hand, a component included in the culture medium as a chelator of micronutrient ions, ethylenediaminetetraacetic acid (EDTA), tends to form complexes with the toxic metals, which decreases their bioavailability and, therefore, their toxicity. This would explain the high EC50 values for Cu and Zn. Natural waters also contain organic substances in solution (dissolved organic matter), composed of humic and fulvic acids, the which form complexes with metal ions. There has been discussion about the inclusion of EDTA in culture media used for algal bioassays when it is desired to evaluate heavy metal toxicity (Geis et al. 2000). However, the Canadian agency protocol suggests including it (Environmental Canada 2007). So also, Domingos et al. (2014) suggest the need to include natural organic and inorganic complexes in the analysis of heavy metal toxicity to ensure adequate environmental protection. Thus, the inclusion of EDTA in the culture medium would bring the results closer to environmental conditions.

Bioassays with native algal strains, isolated from the natural environments of interest, provide more realistic results about the toxicity of substances present in those environments. However, it is convenient to compare those results with those obtained for a standard species under the same test conditions (Carusso et al. 2018). In this work, the EC50s obtained for Cu and Zn metals were higher in the native species than in the standard species (Table 1), which indicates a higher resistance of *S. acutus* to these toxicants. However, the EC10 and EC20 of those substances (Table 1) and of all the binary mixtures analyzed (Table 3), were lower in *S. acutus* than in *R. subcapitata*, which would indicate a higher sensitivity of the native species to low concentrations of the toxicants and their mixtures. According to Beasley et al. (2015), EC10 can be compared to no-effect concentrations (NOECs), i.e. concentrations above which an observable toxic effect would occur. Thus, EC10 could be considered environmentally relevant, as they establish a threshold of protection for organisms.

Heavy metals can affect algal growth in various ways by inhibiting different physiological processes. Photosynthesis, in particular, is a process sensitive to environmental stress. Cu and Zn are essential metals for algae, as they are involved in several physiological processes such as photosynthesis and respiration (Starodub and Wong 1987). However, when these elements are found in high concentrations they can be toxic and inhibit population growth (Franklin et al. 2002). Pb does not play any cellular function, however it can be incorporated to cellular proteins by binding to hydrogen sulfide (SH) groups and can displace other metal ions such as magnesium (Mg), calcium (Ca) and iron (Fe), which are involved in the photosynthetic apparatus and photosynthesis (Lamelas et al. 2009).

The maximum concentrations of Cu, Pb and Zn found in the surface water of Arroyo Burgos, from which the *S. acutus* species strain was isolated, were 0.252 mg/L, 0.176 mg/L and 0.960 mg/L, respectively (Bollani et al. 2019). These concentrations are higher than the EC10 and EC20 obtained for *S. acutus*, but lower than the EC20 obtained for *R. subcapitata*. The higher inhibitory response observed in *S. acutus* to environmental concentrations of heavy metals is indicative of its potential for the assessment of surface water pollution in rural areas by short-term bioassays. *S. acutus* could present adaptive mechanisms to tolerate elevated levels of heavy metals, such as by detoxification or exclusion of these toxicants, as described by some authors for different species of green algae (Janssen and Heijerick 2003; Kalinowska and Pawlik-Skowronska 2010; Piotrowska-Niczyporuk et al. 2012). First, the cell wall plays an important defense role by preventing the entry of heavy metals into the cell, either through adsorption of metal ions on the outer surface or through the release of extracellular ligands (exopolysaccharides) (Sabatini et al. 2009; Kalinowska and Pawlik-Skowronska 2010). In addition, the presence of both intracellular sulfur-rich peptides (phytochelatins) and glutathione favors the development of detoxification mechanisms and reduction of oxidative stress damage caused by essential and non-essential metal ions (Sabatini et al. 2009; Piotrowska-Niczyporuk et al. 2012).

The active ingredient of glyphosate was not toxic to the two algal strains under the conditions and concentrations tested, while *R. subcapitata* experienced significant growth inhibition when confronted with ATANOR glyphosate® at concentrations greater than 10 mg/L (Figure 1). Some evidence would indicate that additives added to herbicide formulations (e.g., surfactants, heavy metals, among others) may be more toxic to algae than the active ingredient of the herbicide in question (González et al. 2019).

The toxicity of the metals Cu, Pb and Zn, and of the glyphosate ATANOR® was greater individually than in the binary mixtures, as shown by the EC50 results, although in the case of *S. acutus* the Cu+Zn mixture had a greater effect than the metals tested individually (Tables 1 and 3). The UT analysis to evaluate the interaction between the substances tested showed that in *R. subcapitata* all the mixtures had UT values>1, indicating an antagonistic relationship between the components of each mixture, even in combination with the glyphosate ATANOR® , evidencing an antagonistic relationship between the herbicide and the metals Cu and Zn (Table 5). The mixture of Cu and Zn for this strain also resulted greater than 1 (UT=2.34). According to Starodub and Wong (1987), in green algae Cu+Zn binary mixtures act antagonistically at concentrations between 0.1 and 0.5 mg/L. In the case of *S. acutus*, a synergistic relationship was observed at low concentrations of the Cu+Pb mixture, similar to the levels found in the surface waters of Arroyo Burgos (Bollani et al. 2019).

Algae are essential components of aquatic ecosystems, major planktonic primary producers and food for other planktonic organisms, fish and invertebrates. The impact of contaminants on algae would directly affect the structure and function of the ecosystem. In the present work, the effects of some pollutants on cultured algal populations in the laboratory were evaluated. It is important to

Note that extrapolation of laboratory results to natural dynamics has its limitations, since the complexity of natural water processes cannot be emulated under the standard conditions used in the tests. For example, culture media do not contain dissolved organic matter, contain large amounts of essential nutrients and have a relatively high pH. Moreover, laboratory bioassays are conducted with a single species, which makes it difficult to establish an effect at the level of natural communities, e.g. natural phytoplankton. However, despite the aforementioned limitations, conducting bioassays using indigenous algal strains, adapted to particular environmental conditions, is more appropriate for inferring possible responses in the natural system. Given its sensitivity to environmental concentrations of heavy metals, the *S. acutus* strain could be incorporated as a new test organism for the analysis of water quality in natural areas. In addition, as it emerges from this work, it is necessary to analyze the effects of interactions between the different substances detected in the water bodies in order to establish more appropriate quality limits for the study area.

Ag�adeciMieuyos. This study was supported by grants UBACyT N° 20020150200116BA and UBACyT N° 20020150200116BA and UBACyT N° 20020150200116BA. 20020130100601BA awarded by the University of Buenos Aires, Argentina.

Refe�eucias

Alloway, B. J. 1995. Heavy metals in soils. Blackie Academic and Professional, London. Pp. 235-274. https://doi.org/10.1007/978-94-011-1344-1.

Aparicio, V. C., E. De Gerónimo, D. Marino, J. Primost, P. Carriquiriborde, and J. L. Costa. 2013. Environmental fate of glyphosate and aminomethylphosphonic acid in surface waters and soil of agricultural basins. Chemosphere **93(9)**: 1866-1873. https://doi.org/10.1016/j.chemosphere.2013.06.041.

Archibald, P. A., and H. C. Bold. 1970. Phycological Studies. XI. The Genus *Chlorococcum* Meneghini. Univ. Texas Public., N7015, Austin, Texas. Pp. 86.

Beasley, A., S. E. Belanger, J. L. Brill, and R. R. Otter. 2015. Evaluation and comparison of the relationship between NOEC and EC10 or EC20 values in chronic *Daphnia* toxicity testing. Environ Toxicol Chem **34**:2378-2384. https://doi.org/10.1002/etc.3086.

Blinova, I. 2004. Use of freshwater algae and duckweeds for phytotoxicity testing. Environ Toxicol **19(4)**:425-428. https://doi.org/10.1002/tox.20042.

Bollani, S., L. de Cabo, C. Chagas, J. Moretton, C. Weigandt, A. Fabrizio de Iorio, and A. Magdaleno. 2019. Genotoxicity of water samples from an area of the Pampean region (Argentina) impacted by agricultural and livestock activities. Environ Sci Pollut Res **26(27)**:27631-27639. https://doi.org/10.1007/s11356-018-3263-9.

Broderius, S. J., M. C. Kahl, G. E. Elonen, D. E. Hammermeister, and M. D. Hoglund. 2005. A comparison of the lethal and sublethal toxicity of organic chemical mixtures to the feathed minnow (*Pimephales promelas*). Environ Toxicol Chem **24(12)**:3117-27. https://doi.org/10.1897/05-094R.1.

Carusso, S., A. B. Juárez, J. Moretton, and A. Magdaleno. 2018. Effects of three veterinary antibiotics and their binary mixtures on two green algae species. Chemosphere **194**:821-827. https://doi.org/10.1016/j.chemosphere.2017.12.047.

Domingos, R. F., A. Gelabert, S. Carreira, A. Cordeiro, Y. Sivry, and M. F. Benedetti. 2014. Metals in the Aquatic Environment-Interactions and Implications for the Speciation and Bioavailability: A Critical Overview. Aquat

Geochem **21(2-4)**:1-27. https://doi.org/10.1007/s10498-014-9251-x.

Duke, S. O. 1988. Herbicides: chemistry, degradation and mode of action. Pp. 1-70 *in* P. C. Kearney and D. D. Kaufman (eds.). Marcel Dekker, USA.

Environmental Canada. 2007. Biological test method: growth inhibition test using a freshwater algae. EPS 1/RM/25, Second Ed. Pp. 53.

Fergusson, J. 1990. The heavy elements. Chemistry, environmental impact and health effects. Pergamon Press, Oxford. Pp 175-182.

Franklin, N. M., J. L. Stauber, R. P. Lim, P. Petocz. 2002. Toxicity of metal mixtures to a tropical freshwater alga (*Chlorella* sp.): the effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. Environ Toxicol Chem **21**(11):2412-2422. https://doi.org/10.1897/1551-5028(2002)021%3C2412:TOMMTA%3E2.0.CO;2. https://doi.org/ 10.1002/etc.5620211121.

Geis, W. S., K. L. Fleming, E. T. Korthals, G. Searle, L. Reynolds, and D. A. Karner. 2000. Modifications to the algal growth inhibition test for use as a regulatory assay. Environ Toxicol Chem **19(1)**:36-40. https://doi.org/10.1002/etc.5620190105.

Guéguen, C., R. Gilbin, M. Pardos, and J. Dominik. 2004. Water toxicity and metal contamination assessment of a polluted river: the Upper Vistula River (Poland). Appl Geochem **19**:153-162. https://doi.org/10.1016/S0883-2927(03)00110-0.

González, D., A. B. Juárez, C. P. Krugn, M. Santos, and S. Vera. 2019. Freshwater periphyton response to technical-grade and two commercial formulations of glyphosate. Ecol Austral **29**:20-27. https://doi.org/10.25260/EA.19.29.1.0.816.

González-Pleiter, M., S. Gonzalo, I. Rodea-Palomares, F. Leganés, R. Rosal, K. Boltes, E. Marco, and F. Fernández- Piñas. 2013. Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: implications for environmental risk assessment. Wat Res **47**:2050-2064. https://doi.org/10.1016/j.watres.2013.01.020.

ISO. 2009. Water quality - Freshwater Algal Growth Inhibition Test with Unicellular Green Algae, revision. International Standardization Organization, Brussels (ISO 8692).

Janssen, C. R., and D. G. Heijerick. 2003. Algal toxicity tests for environ- mental risk assessments of metals. Rev Environ Contam Toxicol **178**:23-52. https://doi.org/10.1007/0-387-21728-2_2.

Jorgensen, S. E., B. H. Sørensen, and H. Mahler. 1997. Handbook of estimation methods in ecotoxicology and environmental chemistry (Vol. 2). CRC Press.

Kahru, A., A. Ivask, K. Kasemets, L. Pollumaa, I. Kurvet, M. François, and H. C. Dubourguier 2005. Biotest and biosensors in ecotoxicological risk assessment of field soils polluted with zinc, lead, and cadmium. Environ Toxicol Chem **24(11)**: 2973-2982. https://doi.org/10.1897/05-002R1.1.

Kalinowska, R., and B. Pawlik-Skowronska. 2010. Response of two terrestrial green microalgae (Chlorophyta, Trebouxiophyceae) isolated from Cu-rich and unpolluted soils to copper stress. Environ Pollut **158**:2778-2785. https: //doi.org/10.1016/j.envpol.2010.03.003.

Kim, R. Y., J. K. Yoon, T. S. Kim, J. E. Yang, G. Owens, and K. R. Kim. 2015. Bioavailability of heavy metals in soils: definitions and practical implementation - a critical review. Environ Geochem Health **37**:1041-1061. https://doi.org/ 10.1007/s10653-015-9695-y.

Küpper, H., I. Setlik, M. Spiller, F. S. Küpper, and O. Prášil. 2002. Heavy metal-induced inhibition of photosynthesis: targets of *in vivo* heavy metal chlorophyll formation. J Phycol **38(3)**:429-441. https://doi.org/10.1046/j.1529-8817.2002.t01-1-01148.x. https://doi.org/10.1046/j.1529-8817.2002.01148.x.

Lamelas, C., J. P. Pinheiro, and V. I. Slaveykova. 2009. Effect of humic acid on Cd (II), Cu (II), and Pb (II) uptake by freshwater algae: kinetic and cell wall speciation considerations. Environ Sci Technol **43(3)**:730-735. https://doi.org/10.1021/es802557r.

Magdaleno, A., L. de Cabo, S. Arreghini, and C. Salinas. 2014. Assessment of heavy metal contamination and water quality in an urban river from Argentina. Braz J Aquat Sci Tech **18(1)**:113-120. https://doi.org/10.14210/bjast.v18n1.p113- 120.

Magdaleno, A., M. Paz, J. Mantovano, L. de Cabo, S. Bollani, C. Chagas, L. Núñez, C. Tornello, and J. Moretton. 2018. Assessment of the impact of rural activities on water quality in the Burgos stream micro-watershed (San Pedro, Buenos Aires Province). Rev Mus Argent Cienc Nat **20(2)**:239-250. https://doi.org/10.22179/REVMACN.20.588.

Nowell, L. H., J. E. Norman, P. W. Moran, J. D. Martin, and W. W. Stone. 2014. Pesticide Toxicity Index-A tool for assessing potential toxicity of pesticide mixtures to freshwater aquatic organisms. Sci Total Environ **476-477**:144-157. https://doi.org/10.1016/j.scitotenv.2013.12.088.

Omar, H. H. 2002. Bioremoval of zinc ions by *Scenedesmus obliquus* and *Scenedesmus quadricauda* and its effects on growth and metabolism. Int Biodeter Biodegr **50**:95-100. https://doi.org/10.1016/S0964-8305(02)00048-3.

Pérez, G. L., A. Torremorell, P. Mugni, P. Rodríguez, M. S. Vera, M. Do Nascimento, L. Allende, J. Bustingorry, R. Escaray, M. Ferraro, I. Izaguirre, H. Pizarro, C. Bonetto, D. P. Morris, and H. Zagarese. 2007. Effects of the herbicide Roundup on freshwater microbial communities: a mesocosm study. Ecol Appl **17**:2310-2322. https://doi.org/10.1890/ 07-0499.1.

Peruzzo, P. J., A. A. Porta, and A. E. Ronco. 2008. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. Environ Pollut **156**:61-66. https: //doi.org/10.1016/j.envpol.2008.01.015.

Pignata, M. L., G. L. Gudiño, E. D. Wannaza, R. R. Plá, C. M. González, H. A. Carreras, and L. Orellana. 2002. Atmospheric quality and distribution of heavy metals in Argentina employing *Tillandsia capillaris* as a biomonitor. Environ Pollut **120**:59-68. https://doi.org/10.1016/S0269-7491(02)00128-8.

Piotrowska-Niczyporuk, A., A. Bajguz, E. Zambrzycka, and B. Godlewska- Zylkiewicz. 2012. Phytohormones as regulators of heavy metal biosorption and toxicity in green alga *Chlorella vulgaris* (Chlorophyceae). Plant Physiol Biochem **52**:52-65. https://doi.org/10.1016/j.plaphy.2011.11.009.

Pizarro, H., M. S. Vera, A. Vinocur, G. Pérez, M. Ferraro, R. M. Helman, and M. dos Santos Afonso. 2015. Glyphosate input modifies microbial community structure in clear and turbid freshwater systems. Environ Sci Pollut Res **23**(6): 5143-5153. https://doi.org/10.1007/s11356-015-5748-0.

Romero, D., M. C. Ríos de Molina, and A. B. Juárez. 2011. Oxidative stress induced by a commercial glyphosate formulation in a tolerant strain of *Chlorella kessleri*. Ecotoxicol Environ Saf **74**:741-747. https://doi.org/10.1016/ j.ecoenv.2010.10.034.

Sabatini, S. E., A. B. Juárez, M. R. Eppis, L. Bianchi, C. M. Luquet, and M. C. Ríos de Molina. 2009. Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. Ecotox Environ Safe **72**:1200-1206. https:
//doi.org/10.1016/j.ecoenv.2009.01.003.

Sarandón, S. J. 2013. Use of agrochemicals in the Province of Buenos Aires. Survey of the use of Agrochemicals in the Province of Buenos Aires - Situation Map and incidences on health. Report of the Ombudsman's Office of the Province of Buenos Aires. Fac Cs Agrarias, UNLP. Pp. 246.

Starodub, M. E. and P. T. S. Wong. 1987. Short-term and long-term studies on individual and combined toxicities of copper, zinc and lead to *Scenedesmus quadricauda*. The Sci Total Environ **63**:101-110. https://doi.org/10.1016/0048-9697(87)90039-8.

Suzuki, S., H. Yamaguchi, N. Nakajima, and M. Kawachi. 2018. *Raphidocelis subcapitata* (=Pseudokirchneriella *subcapitata*) provides an insight into genome evolution and environmental adaptations in the Sphaeropleales. Scientific Reports **8(1)**:1-13. https://doi.org/10.1038/s41598-018-26331-6.

USEPA. 2002. *Selenastrum capricornutum* growth test. *In* Short-term method for estimating the chronic toxicity of effluents and receiving water to freshwater organisms. USA.

Vera, M. S., L. Lagomarsino, M. Sylvester, G. L. Pérez, P. Rodríguez, H. Mugni, R. Sinistro, M. Ferraro, C. Bonetto, H. Zagarese, and H. Pizarro. 2010. New evidences of Roundup Max® (glyphosate formulation) impact on the periphyton community and the water quality of freshwater ecosystems. Ecotoxicology **19**:713-719. https://doi.org/10.1007/s10646- 009-0446-7.