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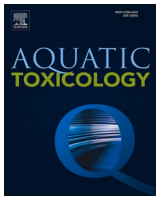

Environmental quality and ecotoxicity of sediments from the lower Salado River basin (Santa Fe, Argentina) on amphibian larvae

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ABSTRACT

The lower Salado River basin receive agricultural, industrial and domestic waste water. So, the aim was to evaluate the quality of three sampling sites that belong to the Salado River basin (S1: Cululú stream; S2: Salado River, at Esperanza City, S3: Salado River at Santo Tomé City) based on physicochemical parameters, metals and pesticides analyses and ecotoxicity on *Rhinella arenarum* larvae. *R. arenarum* larvae (Gosner Stage -GS- 25) were chronically exposed (504h) to complex matrixes of surface water and sediment samples of each site for the determination of the survival rate. Biomarkers of oxidative stress, neurotoxicity and genotoxicity were analyzed in *R. arenarum* larvae (GS. 25) after exposure (96h) to the complex matrix of water and sediment. The water quality index showed a marginal quality for all sites, influenced mainly by low dissolved oxygen, high total suspended solid, phosphate, nitrite, conductivity, Pb, Cr and Cu levels. Metal concentrations were higher in sediment than in water samples (34-35000 times). In total, thirty different pesticides were detected in all water and sediment samples, S1 presented the greatest variety (26). Glyphosate and AMPA were detected in sediments from all sites, being higher in S3. N,N-Diethyl-meta-toluamide (DEET) and atrazine were detected in all water samples. Greatest mortality was observed in larvae exposed to samples from S1 from 288h (43.3%), reaching a maximum value of 50% at 408h. Oxidative stress and genotoxicity were observed in larvae exposed to S1 and S3 matrix samples. Neurotoxicity was observed in larvae exposed to all matrix samples. The integrated biomarker response index showed that larvae exposed to S1 and S3 were the most affected. According to the physicochemical data and the ecotoxicity assessment, this important river basin is significantly degraded and may represent a risk to aquatic biota, especially for *R. arenarum* larvae.

1. Introduction

Pollutants in sediments threaten aquatic life due to the potential accumulation for long periods (Zhang et al., 2021). Aquatic sediments are natural receptors for toxic substances (e.g., metals, organic pollutants, pesticides) that in many cases, are above the accepted limits by international regulations (Duarte-Restrepo et al., 2021; Kilunga et al., 2017). The expansion of urban, agriculture and industrial areas around rivers is a global concern, but mainly, in South America, where native forests and wetlands are being altered and lost due to the increase of those expansions (Cornejo et al., 2020).

Different types of pollutants that derive from these activities may reach aquatic ecosystems. In particular, metals constitute a problem in practically all sediments, regardless their origin (de Groot, 2018). Also, transgenic crops such as soybeans, increased the dependence to chemical pesticides and became one of the most pressing challenges for global

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ecological sustainability and public health (Bonny, 2016). In particular, the lower Salado River basin, which is an affluent of the middle Paraná River, runs through industrial, agricultural and urban regions in the North of Argentina (Gagneten et al., 2007). Previous studies in waters and sediments of this river basin, determined the presence of metals, such as Cr, Cu, Fe and Mn (Gagneten et al., 2007; Gagneten and Paggi, 2009; Gallo et al., 2006), and pesticides, such as glyphosate and its metabolite (aminomethylphosphonic acid; AMPA), atrazine and 2,4-D (Ayarragaray et al., 2014; Reno et al., 2018). Similarly, the Cululú stream, which belongs to the Salado del Norte River basin (Argentina), receives contaminants such as Cr, Pb, Cd from leather, metallurgy, electroplating, glazing, mirroring, agriculture, dairy (among others) industries (Zilli and Gagneten, 2005). Studies that evaluate the effects of the pollution of the lower Salado River basin on aquatic organisms include analyses of benthos and zooplankton communities' structures (Gagneten and Paggi, 2009; Marchese et al., 2008; Zilli and Gagneten, 2005). Other investigations use multiple biomarkers (detoxification and oxidative stress: antioxidant enzymes, lipid peroxidation) in liver, gills and kidney of the fish *Prochilodus lineatus* in order to evaluate changes in water quality (Cazenave et al., 2009). The high pollution due to presence of metal and agrochemicals, low dissolved oxygen, and abrupt increases in temperature directly affect the inhabiting fish populations, causing massive mortality across the river basin. There is also no available information about the effects on other organisms related to this river basin, such as amphibians.

As mentioned above, sediments of aquatic system are the sink of several atmospheric and terrestrial pollutants (especially pesticides). Several studies have shown that polluted sediments cause mortality and decrease growth and survival rates on amphibian larvae (Lanctôt et al., 2017; Sparling et al., 2006). It is important to note that amphibian larvae interact strongly with sediments during their early stages (Peltzer et al., 2013). Larvae of amphibians are benthic organisms that are constantly in contact with sediments. They ingest organic and inorganic particles from the sediment surface, so they are exposed to contaminants through their dermis and the gastrointestinal route (Sansiñena et al., 2018). Benthic organisms also represent a significant part of the food chain for groundfish and other vertebrates. So, they are crucial organisms for the trophic transfer and biomagnification of pollutants (Ding et al., 2022). In consequence, benthic organisms are indicators of sediment quality. In Argentina, the presence of pollutants in sediments of the Paraná River basin affected the survival, growth, development, and reproduction of amphibians (Peltzer et al., 2013; Peluso et al., 2022). For this reason, the integrated analysis of physicochemical and ecotoxicological parameters provide relevant and realistic information about the environmental quality and its real consequences for amphibian populations (Peluso et al., 2022). In particular, *Rhinella arenarum* is a species of toad, native of Argentina and widely used in toxicity bioassays (Lajmanovich et al., 2019; Peluso et al., 2022; Pérez Coll et al., 2017). Despite its conservation status is classified as "least concern" according to the International Union for Conservation of Nature Red List of Threatened Species (Kwet et al., 2004), previous studies warn about the vulnerability of this species to changes in the environment (Babini et al., 2015).

Studies of biological responses on test organisms are early alerts of polluted environments and allow to characterize the ecological risk for amphibian larvae (Peltzer et al., 2008). Therefore, the use of multiple biomarkers in amphibians has gained increasing interest in the last decades due to their ecological relevance when understanding the effect of one or a mixture of contaminants (Venturino et al., 2003; Yologlu and Ozmen, 2015). In addition, due to their sensitivity, oxidative stress biomarkers, such as catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), reduced glutathione (GSH), and lipid peroxidation (TBARS) levels are frequently used to asses toxic effect in amphibians exposed to different matrixes (Brix et al., 2022; Cheron et al., 2022; Falfushinska et al., 2008; Maldonado-López et al., 2022; Peluso et al., 2021). Several contaminants, including metals, pesticides, and aromatic chemicals, can increase oxidant concentrations and produce reactive oxygen species (ROS) (Brix et al., 2022; Cheron et al., 2022). Also, pollutants can induce DNA damage through direct or indirect pathways. The micronucleus test is commonly used in amphibians as a genotoxicity biomarker (Lajmanovich et al., 2005). Neurotoxicity biomarkers, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are widely employed in amphibians to assess the toxic effects of substances (Lajmanovich et al., 2011).

The aim of the present study was to evaluate the lethal and sublethal toxicity (biomarkers of oxidative stress, neurotoxicity and genotoxicity) of a complex matrix of sediment and water from the lower Salado River basin on *Rhinella arenarum* larvae, a native amphibian species. Also, physicochemical parameters, metals and pesticide residues were analyzed.

2. Material and methods

2.1. Study area and sampling of water and sediment

Samples were taken at three different sites through the Salado River basin, on the east center of Santa Fe, Argentina (Fig. 1). Site 1 (S1 = 31°21′47.4"S 60°56′48.3"W) was at Cululú stream, a tributary of Salado River basin. This stream receives pollutants from the leather industry, metallurgy, electroplating, glazing, mirror industry, agriculture, dairy farms, etc., which affect the quality of its bottom sediments. Site 2 (S2 = 31°23′04.7"S 60°53′58.1"W) and Site 3 (S3: 31°39′26.0"S 60°45′21.8"W) were located on the lower Salado River. S2 was sampled at the coast of the municipal camping of Esperanza City, and S3 was sampled at the coast of the beach of Santo Tomé City. These cities are the two most important of the Salado River lower basin and receives pollutants of diverse origin (urban, industrial and agricultural).

Sediment and complex surface water samples were collected in March 2022. Sediment samples (2 kg) were collected according to ASTM (2014) in plastic containers (previously washed with HCl 10%, acetone and abundant deionized water) and water samples (10 L) were collected according to Alberro et al. (2011) in clean plastic bottles (washed with HCl 10%, pure acetone and abundant deionized water). All samples were stored at 4°C in dark and transported immediately to laboratory.

2.2. Physicochemical parameters (in situ and in laboratory), metals and pesticides

In the sampling sites, temperature, dissolved oxygen (DO), conductivity, pH, and turbidity were measured with a multi-water quality checker HORIBA U-50.

In laboratory, total suspended solids (TSS) and biological oxygen demand (BOD5) were determined in water according to APHA (2012). Chemical oxygen demand (COD, method No. 8000), ammonium (method No. 8155), nitrates (method No. 8192), nitrites (method No. 8507), phosphates (method No. 8048), chlorides (method No. 8113) and sulphates (method No. 8051) were measured following the HACH protocols with a HACH DR-1900 spectrophotometer. Organic matter (OM) was determined by the method proposed by Dean (1974) in sediment samples, The pH and conductivity were measured from suspensions with a 1:2 ratio (soil and water, respectively) with a conductimeter ADWA AD204 and a pH meter ADWA AD-12.

Metals were measured by Total Reflection X-ray fluorescence (TXRF) S2 Picofox, Bruker with a molybdenum tube. For water samples, 10 μL of Ga 100 mg/L was added as internal standard to 1 mL of sample. Then, it was shaken in a vortex and 5 μL of the solution was deposited in a quartz reflector and dried under an infra-red lamp. Finally, samples were measured in the spectrometer for 300 s. For sediment samples, a suspension containing 0.25 g of the sediment sample and 10 mL of agarose solution 0.1% was prepared in a falcon tube. Then, 100 μL of Ga 1000 mg/L was added as internal standard, homogenized in vortex. Finally, 5

Fig. 1. Study area showing the sampling sites around the lower Salado River basin: S1: Cululú Stream; S2: Municipal Camping of Esperanza City, S3: Municipal Beach of Santo Tomé city. The map was personally made with maps from Instituto Geográfico Nacional (https://mapa.ign.gob.ar).

μL of the suspension was deposited in a quartz reflector and dried under infra-red lamp and measured in the spectrometer for 300 s. The limit of detection (LOD) and the limit of quantification (LOQ) for each measured metal are presented in the result section (Table 2).

A screening of pesticides was performed by UHPLC-MS/MS (Waters). The sediment and water samples were distributed into two aliquots, the first to determine glyphosate and AMPA following the methodology reported by Aparicio et al. (2013) and the second to perform the analysis of multiple pesticide residues following the procedure reported by De Gerónimo et al. (2015). The limit of detection (LOD) and the limit of quantification (LOQ) for each measured molecule are presented in the result section (Table 3).

2.3. Test organisms

Rhinella arenarum adults were collected from a low impact area (S 34°49′53.8′′ W 58°6′17.08′′). The collection was allowed by the Dirección de Flora y Fauna from Buenos Aires Province (Res. 01133069). The embryos of *R. arenarum* were obtain following the AMPHITOX protocol (Pérez Coll et al., 2017). Individuals were kept in AMPHITOX Solution (AS, concentration in mg/L: Na^+ 14.75, Cl^- 22.71, K^+ 0.26, Ca_2^+ 0.36 and HCO_3^- 1.45), ensuring oxygen availability. The embryonic and larval stages were defined according to (Gosner, 1960). The handling of the animals was carried out according to the regulations for the use of amphibians in laboratory research (Beaupre et al., 2004), approved by CICUAE-UNSAM (Res. 1/2022) and the Dirección de Flora y Fauna from Buenos Aires Province (Res. 01133069).

2.4. Chronic toxicity bioassays

Grass and roots were removed from sediment samples. Chronic (504 h) toxicity bioassays were performed following the standardized ASTM (2007) protocols with some modifications. Briefly, for each site a complex matrix with sediment and water of each site were place in 500-mL flask. Two hundred mL of sediment samples were placed in the flask to obtain a 2-cm homogeneous layer with 250 mL of water samples from each site, reaching a water column of 2 cm. A silica inert sediment and AS were used as control (Chapman, 1988; Peluso et al., 2022). Treatments were performed in triplicate. Larvae were feed with 3 granules of fish food (TetraColor®) per flask every other day. Sediments were allowed to settle for 24 h and 10 larvae (S.25) was added to each flask. Water samples were partially renewed (100 mL) every other day with the help of a syringe to ensure the DO levels in each experimental unit. Conductivity, pH and dissolved oxygen were measured every 48 h in the water of each test chambers during the bioassay in order to ensure the conditions. Temperature (20±2°C) and the light/dark cycle (16/8 h) were maintained constant.

Lethality was evaluated every day; dead individuals were removed, and individuals were observed in order to analyze sublethal morphological effects.

2.5. Biomarkers of oxidative stress and neurotoxicity

At the same time, with the lethality, a test for the determination of biomarkers of oxidative stress and neurotoxicity was carried out, 50 larvae (S.25) were placed, in triplicate, with 200 mL of sediment samples (or control substrate) and 250 mL of water samples from the sites (or AS) on 500 mL-flasks. Treatments were allowed to settle 24 h. Temperature (20 ± 2 °C) and the light/dark cycle (16/8 h) were maintained constant. After exposure (96 h), individuals were collected, washed with deionized water, dried with absorbent paper and frozen until determinations.

The homogenates were performed with a KCl 154 mM solution containing benzamidine 0.2 mM and phenylmethylsulfonyl fluoride 0.5 mM. Then, homogenates were centrifuged for 20 min at 10,000 g and 4°C. Determinations were performed on the supernatants. Protein levels were measured following the Bradford (1976) protocol. Catalase (CAT) activity was determined according to Lück (1965) by calculating the amount of enzyme necessary to dismutate 1 mmol of hydrogen peroxide for 1 min. Its activity was expressed as U CAT (mmol*min)/mg protein (molar extinction coefficient: 40/M × cm). Glutathione S-transferase (GST) activity was estimated as the amount of enzyme required to catalyze the conjugation of 1 mmol of GS-DNB per min (Habig et al., 1974). Its activity was expressed as U GST mg (mmol*min)/protein (molar extinction coefficient: 9.6/mM × cm). The content of reduced glutathione (GSH) was measured by deproteinization of homogenates with 10% trichloroacetic acid, followed by centrifugation for 10 min at 10000 × g and 4°C. The supernatants were incubated with 6 mM 5,

5-dithiobis-(2-nitrobenzoic) acid and 143 mM sodium phosphate buffer pH 7.5, 6.3 mM EDTA (Anderson, 1985). The concentrations were expressed as nmol GSH/mg protein. Finally, lipid peroxidation was determined as the thiobarbituric acid reactive substances (TBARS) method (Buege and Aust, 1978) and the concentrations were expressed as nmol TBARS /mg protein.

Butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) activities were determined according to Ellman et al. (1961), using buffer tris 25 mM (pH 8), DTNB 0.3 mM, acetylthiocholine or butyrylthiocholine iodide (2 mM) and the homogenate. Molar extinction coefficients of 13.6/mM × cm and 14.15/ mM × cm were employed for the calculation of BChE and AChE activities, respectively. The activities were expressed as U BChE (nmol*min)/mg protein) and U AChE (nmol*min)/mg protein).

All reagents were of analytical grade.

2.6. Biomarker of genotoxicity: Micronuclei frequency

For the determination of the micronuclei frequency (MN frequency), five larvae (S.28) were place into the complex matrix with sediment and water of each site form in a 500 mL-flasks at 96 h in, in triplicate. The matrix consisted of 200 mL of sediment samples and 250 mL of water samples from each site. A control group with 200 mL of the inert substrate and 250 mL of AS was performed. Treatments were allowed to settle for 24 h. After the exposure, larvae were cleaned, numbed with ice, and a blood sample was taken from each larva and place on slides. Slides were air dried, fixed for 20 min with cold methanol and then for 3 min with May Grünwald. Finally, they were stained with filtered Giemsa solution (10%) (18 min) (Cabagna et al., 2006). The MN frequency was determined by examining 1,000 mature erythrocytes following the Fenech (2000) criteria under a Leica DFC7000 T microscope.

2.7. Data analysis

For each site, a water quality index (WQI) was calculated as proposed by Neary et al. (2001). The obtained values were compared to an environmental objective (EO). The EO for metals and physicochemical parameters (Table 1) were values recognized as limit for the protection of aquatic life by the Argentinean the Law number 24,051 (831/93) and Ávila Pérez et al. (2011). The parameters included in the analysis were: pH, OD, conductivity, BOD5, TSS, phosphate, ammonium, nitrate, nitrite, Pb, Cr, Cu and As. Pesticide values were not employed in the index since there are no limits for all the detected pesticides. The classification of the WQI was very poor (0-29), poor (30-44), marginal (45-64), bad (65-79), good (80-94) and excellent (95-100).

All comparisons and graphs were made with GraphPad Prism 8. The biomarkers' values were compared using one -way ANOVA followed by a Dunnett's test when the assumptions of homoscedasticity and normality were fulfilled and when these requirements were not fulfilled, a Kruskall-Wallis followed by a Dunn post test was done.

A multi-level integrated biomarker response (IBR) index was calculated according to Devin et al. (2014). This IBR is based the proposed by Beliaeff and Burgeot (2002) and Samanta et al. (2018). Briefly, biomarker values were standardized (Y) and score values (S) were estimated as follows: $S = Y + |\min|$, where $S \geq 0$ and $|\min|$ is the absolute minimum value of each biomarker response. The S values of oxidative stress data (CAT, GST, GSH and TBARS) were used to calculate the area of the radar diagram following equation 1. Then, the area value of the oxidative stress star plot was used as the S values to calculate the multi-level IBR index, which included the S values for the BChE and AChE activities and the MN frequency. The IBR were calculated as the total area of the radar diagram, which was calculated by defining two successive biomarkers following the equation 1:

$$A_i = S_i + S_{i+1} * \sin(2\pi / k)/2$$

Where k is the number of the determined biomarkers.

Table 1
Physicochemical parameters measured *in situ* and in laboratory in water and sediment samples from Cululú stream (S1); Salado River at Esperanza Camping (S2) and Salado River at Santo Tomé Beach (S3). EO: Environmental objective; DO: Dissolved Oxygen; BOD5: Biological Oxygen Demand 5; TSS: Total Suspended Solids; COD: Chemical Oxygen Demand; na: Not available. "*" Not compliant with the EO.

Water	Units	Site 1	Site 2	Site 3	EO
In situ					
Temperature	°C	22.14 ±0.03	23.45 ±0.08	23.53 ±0.09	na
pH		7.94±0.19	7.71±0.04	7.46±0.16	5-9
Conductivity	mS/cm	2.74 ±0.36*	1.80 ±0.06*	1.57±0.23*	1.25
Turbidity	NTU	281±18	317.66 ±5.85	293.66 ±22.74	na
DO	mg/L	5.46±0.44	3.26 ±1.01*	2.66±0.35*	5.5
Total dissolved solids	g/L	1.78±0.19	1.17±0.03	0.97±0.12	na
In laboratory					
Ammonium	mg/L	0.32±0.03	0.26±0.02	0.25±0	1.29
Nitrate	mg/L	0.650 ±0.07	0.055 ±0.007	0.140 ±0.02	17
Nitrite	mg/L	0.06 ±0.005*	0.040 ±0.001	0.087 ±0.01*	0.06
Phosphate	mg/L	3.59±1.2	3.13±0.07	2.08±0.03	na
Sulphate	mg/L	62.5±2.1	61±1	65±1	na
Chloride	mg/L	204±11.31	151±25	109.5±7.7	na
BOD5	mg/L	6.72	6.38	6.34	11.63
TSS	mg/L	110*	132*	120*	81.25
COD	mg/L	29.3±1.5	25.07 ±2.58	31.66 ±1.52	na
Sediments					
pH		8.90±0.2	8.70±0.3	8.25±0.04	
Conductivity	mS/cm	3.21±0.01	2.34±0.2	3.41±0.03	
Organic matter	%	5.26±1.2	6.32±0.12	5.12±0.31	

Equation 1. . Formula to calculate the area of the triangle defined by two successive biomarkers in a k biomarker study.

For each site, six IBR were calculated, by implementing a permutation matrix that gives different order of biomarkers for the radar diagram in order to avoid misinterpretation of the results. The IBR values obtained for each site were statistically compared with a Kruskal-Wallis test followed by a Dunn's post-test since the distribution was not normal.

3. Results

3.1. Physicochemical analysis in situ and in laboratory

Table 1 shows the physicochemical parameters of each sampling site. Conductivity was particularly high in S1 and in all sites exceeded the established EO. Dissolved oxygen was lower than its EO in S2 and S3. The TSS levels were higher than its EO in all sites. Nitrite levels were higher than the EO in S1 and S3. Phosphate and sulphate levels were high in all sites and COD was higher than BOD5 for all cases. The levels of conductivity and pH were higher in sediments in comparison to water samples.

3.2. Metal analysis

Table 2 shows the levels of metals of each sampling site. The levels of Ti were higher in S3. The levels of Cr exceeded the EO in S2. The levels of Fe increased along the course of the river. The levels of Cu and Zn exceeded the EO in all sites, and in particular in S1 its concentration was higher than in the other sites. The levels of Pb were higher than the EO in S2 and S3. In general, metal concentrations were from tenths to thousands of times higher in sediment in comparison to water samples.

Table 2
Metal concentration in water and sediment samples from Cululú stream (S1), Salado River at Esperanza Camping (S2) and Salado River at Santo Tomé Beach (S3). "-": Not detected; na: Not available; values with "<": detected but under the quantification limit; LOD: Limit of detection; LOQ: Limit of quantification; EO: Environmental objective. "*" Not compliant with the EO.

Water (mg/L)							Sediment (mg/kg)				
	LOD	LOQ	Site 1	Site 2	Site 3	EO	LOD	LOQ	Site 1	Site 2	Site 3
K	0.1	0.2	20	11	11	na	5	10	8225	9389	10547
Ca	0.1	0.2	47	26	24	na	5	10	6757	5385	5669
Ti	0.01	0.02	0.026	0.091	0.13	na	2	4	2305	2209	2474
V	0.01	0.02	0.032	0.014	0.016	0.1	2	4	34	26	30
Cr	0.002	0.01	-	<0.01	-	0.002	2	4	44	119	86
Mn	0.005	0.01	0.023	0.01	0.014	0.1	2	4	488	235	384
Fe	0.005	0.01	0.42	1.4	2.1	na	2	4	14561	10516	14119
Ni	0.002	0.01	<0.01	<0.01	0.023	0.025	1	2	18	63	36
Cu	0.002	0.01	0.044*	<0.01*	0.011*	0.002	1	2	13	-	12
Zn	0.002	0.01	0.094*	0.068*	0.039*	0.03	1	2	35	23.2	34
As	0.002	0.01	0.031	0.028	0.024	0.05	0.6	1.2	3.4	1.3	3.8
Sr	0.002	0.01	1.1	0.47	0.41	na	1	2	144	134	158
Ba	0.03	0.06	0.1	0.18	0.05	na	5	10	253	301	316
Pb	0.002	0.01	-	<0.01*	<0.01*	0.001	1	2	9	9	10

Table 3
Summary of biocide residues and their metabolites concentrations in water a- sediment samples from Cululú stream (S1), Salado River at Esperanza Camping (S2) a-Salado River at Santo Tomé Beach (S3). LOD: Limit of Detection; LOQ: Limit of quantification; values with "<": detected but under the quantification limit.; -: not detected

Compound	Chemical group	Water (μg/L)						Sediment (μg/kg dry weight)					
		LOD (μg/L)	LOQ (μg/L)	Detection Frequency (%)	S1	S2	S3	LOD (μg/Kg)	LOQ (μg/Kg)	Detection Frequency (%)	S1	S2	S3
INSECTICIDES													
Chlorpyrifos-Methyl	Organophosphorous	0.002	0.005	100	<0.005	<0.005	<0.005	0.07	0.30	33.3	<0.30	-	-
Pirimiphos methyl	Organophosphorous	0.002	0.005	33.3	<0.005	-	-	0.05	0.20	0	-	-	-
Diazinon	Organophosphorous	0.001	0.004	100	<0.004	<0.004	<0.004	0.07	0.40	0	-	-	-
Dimethoate	Organophosphorous	0.001	0.003	66.7	<0.003	<0.003	-	0.07	0.50	33.3	<0.50	-	-
Fipronil	Phenylpyrazole	0.001	0.003	100	<0.003	<0.003	<0.003	1.80	6.10	0	-	-	-
Carbofuran	Carbamate	0.002	0.006	66.7	<0.006	-	<0.006	0.05	0.20	0	-	-	-
Pirimicarb	Carbamate	0.001	0.003	66.7	0.008	<0.003	-	0.50	1.50	33.3	<1.50	-	-
Imidacloprid	Neonicotinoid	0.003	0.008	100	0.039	0.054	0.058	0.07	0.40	0	-	-	-
DEET	N.N-dialkylamides	0.002	0.005	100	0.013	0.011	0.013	0.07	0.30	66.7	36.30	24.30	-
Piperonyl butoxide	Not qualified.	0.0007	0.0025	100	0.003	0.023	0.009	0.07	0.30	66.7	<0.30	<0.30	-
HERBICIDES													
Glyphosate	Organophosphonates	0.05	0.10	0	-	-	-	0.30	0.80	100	10.0	21.2	81.3
AMPA	Organophosphonates	0.08	0.15	33.3	2.2	-	-	0.40	1.40	100	17.0	53.4	46.2
2.4-D	Phenoxyacetic acid	0.005	0.015	0	-	-	-	1.50	3.50	33.3	6.00	-	-
Fomesafen	Diphenyl ethers	0.003	0.009	100	0.011	<0.009	0.010	0.07	0.50	0	-	-	-
Acetochlor	Chloroacetamide	0.003	0.009	66.7	-	0.036	0.037	0.50	1.60	0	-	-	-
Atrazine (ATZ)	Triazine	0.001	0.004	100	0.062	0.036	0.045	0.05	0.20	66.7	<0.20	<0.20	-
ATZ-OH	Triazine	0.003	0.009	100	0.050	0.023	0.019	0.07	0.50	100	<0.50	<0.50	<0.50
ATZ-desethyl	Triazine	0.0004	0.002	100	<0.002	<0.002	<0.002	0.60	2.10	0	-	-	-
ATZ-Desisopropyl	Triazine	0.002	0.006	33.3	-	-	0.002	0.80	3.00	0	-	-	-
Ametryn	Triazine	0.001	0.003	33.3	<0.003	-	-	0.05	0.20	0	-	-	-
Metsulfuron Methyl	Sulfonylurea	0.002	0.006	66.7	-	<0.006	<0.006	0.05	0.20	0	-	-	-
Chlorimuron ethyl	Sulfonylurea	0.003	0.007	100	<0.007	0.011	<0.007	0.50	1.60	0	-	-	-
Diclosulam	Triazolopyrimidines	0.002	0.006	33.3	<0.006	-	-	0.07	0.50	0	-	-	-
Imazethapyr	Imidazolinone	0.001	0.004	100	0.011	0.008	<0.004	0.08	0.30	0	-	-	-
Imazapyr	Imidazolinone	0.001	0.004	100	<0.004	<0.004	<0.004	0.50	1.60	0	-	-	-
Imazaquin	Imidazolinone	0.0003	0.001	67.7	-	<0.001	<0.001	0.70	0.50	0	-	-	-
FUNGICIDES													
Epoxiconazole	Triazol	0.001	0.002	100	<0.002	<0.002	<0.002	0.05	0.80	0	-	-	-
Tebuconazole	Triazol	0.002	0.005	100	0.021	0.006	<0.005	0.05	0.20	0	-	-	-
Metconazole	Triazol	0.002	0.006	100	<0.006	<0.006	<0.006	0.05	0.20	0	-	-	-
Metalaxyl	Acylalanin	0.001	0.004	100	<0.004	<0.004	<0.004	0.07	0.30	33.3	<0.30	-	-
Total number of pesticides detected:					24	22	22				11	6	3

Total number of biocides detected per site: S1: **26**, S2: **24**, S3: **23**
Total biocides detected in lower Salado River basin: **30**

3.3. Pesticides analysis

In all sites, pesticides were detected in water and sediment samples (Table 3). Mainly, pesticides were detected in water samples, and S1 presented the greatest variety. Glyphosate and AMPA were detected in sediments from all sites, and S3 presented the highest concentration of glyphosate. High DEET concentrations were detected in all samples, being higher in sediment samples from S1 and S2. Also, atrazine and its metabolites were detected in all sites, mainly in water samples. The ranges of total measured biocides concentrations in water and sediments were, 0.003–2.2 μg/L and 6–81.3 μg/kg dry weight, respectively.

3.4. Water quality index (WQI)

According to the WQI, all sites showed a marginal water quality. In particular, S2 presented the lowest value (52.9), followed by S1 (54) and S3 (54.9). The WQI values were influenced mainly by the low DO levels, the high TSS, phosphate, nitrite, conductivity, Pb, Cr and Cu levels.

3.5. Chronic toxicity bioassay

Significant mortality was observed in larvae exposed to the complex matrix of water and sediment sample from S1 at288 h. Mortality reached a maximum value of 50% at 408 h of exposure in the S1 treatment. No significant mortality was observed in the control group and the complex matrix of water and sediment from S2 and S3 (Fig. 2) at all exposure times.

Also, no morphological alterations were observed in larvae exposed to any treatment.

3.6. Biomarkers of oxidative stress

Oxidative stress was observed in larvae exposed to the complex matrix of water and sediment samples from S1 and S3 (Fig. 3). The activity of CAT, GST, and GSH and TBARS levels were significantly higher in larvae exposed to complex matrix of water and sediment samples from S1 in comparison to the control (Fig. 3 A-D). The activity of GST, and the GSH and TBARS levels were higher than the control group in larvae exposed to complex matrix of water and sediment samples from S3 (Fig. 3 B-D).

3.7. Biomarker of neurotoxicity

The BChE activity was lower in larvae exposed to the complex matrix of water and sediment samples from S2 and S3 than the control group (Fig. 4 A). On the other hand, the AChE activity was higher in larvae exposed to all treatments in comparison to the control (Fig. 4 B).

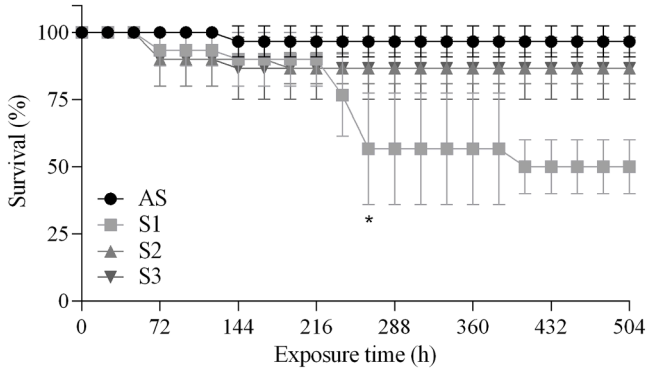

Fig. 2. Survival (%) of *Rhinella arenarum* larvae (S.25) exposed for 504 h to the control (AS, AMPHITOX solution and inert substrate) and water and sediment samples from Cululú stream (S1), Salado River at Esperanza Camping (S2) and Salado River at Santo Tomé Beach (S3). "*" Significant differences with control AS, $p < 0.05$.

3.8. Biomarker of genotoxicity

A significantly higher MN frequency was observed in larvae exposed for 96 h to the complex matrix of water and sediment samples from S1 and S3 in comparison to the control group (Fig. 5 A). Illustration of a control erythrocyte and erythrocytes with MN from a larvae exposed to samples from S1 and S3 are shown in Fig. 5 B, C and D, respectively.

3.9. Integrated biomarker response (IBR) index

Two examples of radar diagrams for each site are shown in Fig. 6 A and B. In comparison to the mean IBR index value of control group (0.02 ±0.11), S1 and S3 presented significant differences ($p<0.01$). S1 presented the highest mean IBR value (18.55±2.37) followed by S3 (13.19 ±1.71) and S2 (7.64±1.79) (Table 4).

4. Discussion

Field and laboratory studies allow to identify factors that lead to the toxic effects; however, evidence for local solutions of aquatic system contamination as a whole complex system (water-sediment) are scare (Grant et al., 2016). In the Salado River lower basin, a low environment quality was recorded. In particular, DO levels were lower in water samples from S2 and S3 (1.7 and 2.06 times, respectively) than the limit for protection of aquatic life (5.5 mg/L) (Ossana et al., 2016). All sites presented high conductivity, turbidity and total dissolved solids, which are characteristic of this river basin (Gagneten et al., 2007). Previous studies reported similar results and suggested that the lower Salado River is characterized by hard waters, with high conductivity and suspended material (Cazenave et al., 2009; Gagneten et al., 2007; Marchese et al., 2008). The levels of phosphate were higher than the previously reported in freshwater habitats from similar agricultural areas and can negative affected amphibians' development and survival (Peltzer et al., 2008). Inorganic phosphate is included in agrochemicals such as fertilizers and may lead to eutrophication in ponds, which constitute a threat to aquatic life, mainly for amphibians (Bishop et al., 1999).

Previously, Gagneten et al. (2007) and Marchese et al. (2008) also registered high concentrations of metals such as Cr, Cu, Pb, Cd in water and sediments of the Salado River lower basin. However, this information is not up to date. In our study, the levels of Cr in S2, close to Esperanza city, exceeded the limit for protection of aquatic life (0.002 mg/L) according to the Argentine Law 24.051, decree 831/93. A previous study informed higher concentration of this metal in the Cululú Stream and in the Salado River, with a maximum value of 4.57 mg/L at a site close to S2 (Gallo et al., 2006). These finding alerts that, besides the historical and background Cr pollution already recorded by the authors more than a decade ago, there is also a current input of Cr in water, which comes from industrial activities and persists due to the lack of controls. Also, the levels of Cu and Zn exceeded their corresponding limits (0.002 and 0.03 mg/L, respectively). Indeed, the levels of Cu in S1 were higher than the 168 h-LC50 (0.019 mg/L) for *R. arenarum* embryos. In all sites, the levels of Cu were higher than the NOEC of sublethal effects (0.0075 mg/L) for *R. arenarum* embryos (Aronzon et al., 2011). Also, the levels of Pb were higher than the limit for protection of aquatic life (0.001 mg/L) in S2 and S3. Metals were concentrated on sediment samples from all sites, which may constitute a potential threat to aquatic organisms that are chronically exposed to them. The high levels of metals detected and the interactions between them and/or other parameters may constitute a risk for the amphibians that inhabit those areas (Khangarot and Ray, 1987).

A total of thirty were detected in at least one of the environmental compartments in sediments and surface waters from the three sites of the lower Salado River basin (12 herbicides with 4 metabolites, 10

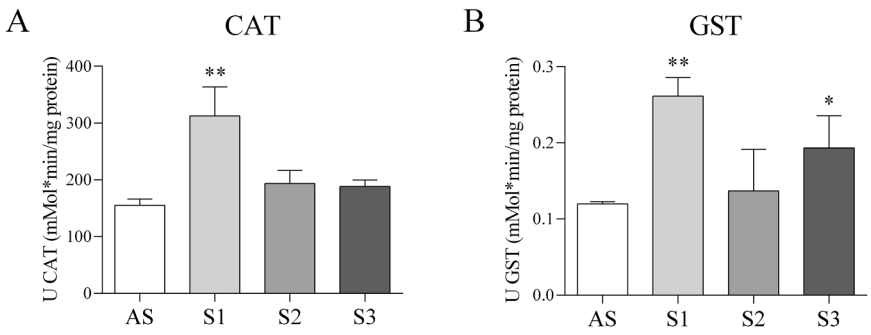

Fig. 3. Catalase -CAT- (A), glutathione S transferase -GST- (C) activities, and reduced glutathione -GSH- (D) and lipid peroxidation -TBARS- (E) levels in *Rhinella arenarum* larvae (S.25) exposed for 96 h to the control AS (AMPHITOX solution and inert substrate) and treatments (water and sediment samples) from all sites: Cululú stream (S1), Salado River at Esperanza Camping (S2) and Salado River at Santo Tomé Beach (S3). Data is presented as mean ± SD. "*": Significant differences with the control p<0.05; "**": Significant differences with the control p<0.01.

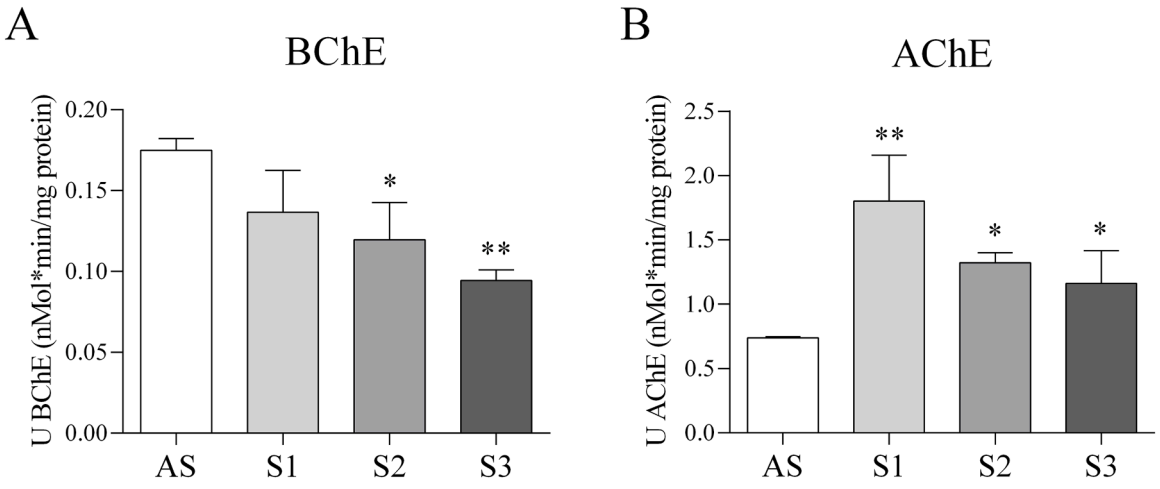

Fig. 4. Butyrylcholinesterase -BChE- (A) and acetylcholinesterase (B) activities in *Rhinella arenarum* larvae (S.25) exposed for 96 h to the control AS (AMPHITOX solution and inert substrate) and treatments (water and sediment samples) from all sites: Cululú stream (S1), Salado River at Esperanza Camping (S2) and Salado River at Santo Tomé Beach (S3). Data is presented as mean ± SD. "*": Significant differences with the control p<0.05; "**": Significant differences with the control p<0.01.

insecticides and 4 fungicides). To our knowledge, this is the first study to report the presence of such a high number of biocides in water and sediments of the lower Salado River. Pesticide concentrations were slightly lower than those reported in water from a basin in the center of the province of Buenos Aires (0.00 - 5.49 μg/L) and similar in sediments (2.60 -72.90 μg/kg) (Pérez et al., 2021). The great variety of detected pesticides indicates their widespread presence in the water and sediment of this basin where the risk of chronic exposure of living organisms to biocide mixtures is unavoidable.

Atrazine-OH was a ubiquitous compound in both environmental compartments and glyphosate was quantitatively the most relevant, as have been previously reported in other aquatic ecosystem of the region (Alonso et al., 2018). Among the detected pesticides, carbofuran and imazapyr, are the most hazardous for aquatic organism as they are classified as Class I (Highly Toxic) while pesticides as chlorpyrifos-methyl, diazinon, dimethoate, fipronil, imidacloprid, are classified as Class II, moderately toxic). As has been determined by numerous studies (Aparicio et al., 2013; Etchegoyen et al., 2017; Pérez et al., 2021), in Argentina, agricultural activity is the principal source of contamination due to the massively use of pesticides, which are transported to aquatic ecosystems and impact their quality. Sequences with long fallow periods, related to high participation of soybean crops and low crop residues, must be avoided in order to lower runoff process and agrochemicals mobility (Kraemer et al., 2022). Data on pesticides residues of the lower Salado River in the Province of Santa Fe are scarce. Ayarragaray et al. (2014) registered in urban and rural drainage channels in San Justo city, glyphosate (0.015-0.89 ng/L and 8.0-49.1 μg/kg) and AMPA (0.5-4.0 μg/L and 8.0-60.7 μg/kg) in water and sediments, respectively. Reno et al. (2018) recorded atrazine (1.028 μg/L) and 2, 4-D (2.244 μg/L) in the same river. Moreover, during a recently

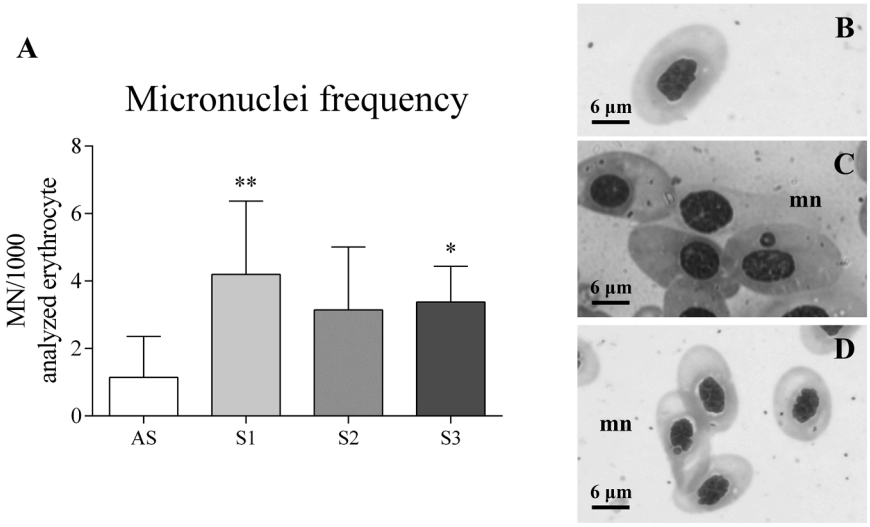

Fig. 5. Micronuclei (MN) frequency in *Rhinella arenarum* larvae (S.25) exposed for 96 h to the control AS (AMPHITOX solution and inert substrate) and treatments (water and sediment samples) from all sites: Cululú stream (S1), Salado River at Esperanza Camping (S2) and Salado River at Santo Tomé Beach (S3) (A). Control erythrocyte (B) and erythrocytes with micronucleus from larvae exposed to samples from S1 (C) and S3 (D). Data is presented as mean ± SD. "*": Significant differences with the control p<0.05; "**": Significant differences with the control p<0.01.

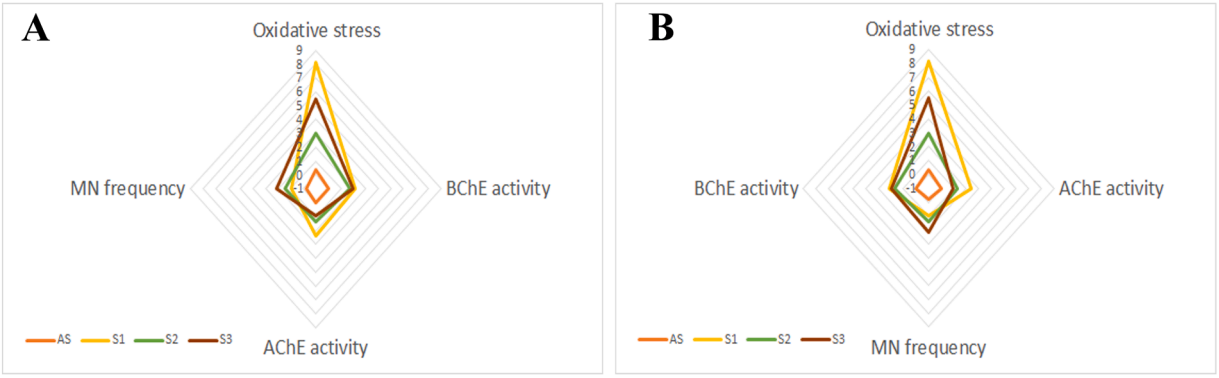

Fig. 6. Star plots examples for two permutations (**A** and **B**) for calculating the integrated biomarker index (IBR) for *Rhinella arenarum* larvae exposed to the control AS (AMPHITOX solution and inert substrate) and treatments (water and sediment samples) from all sites: Cululú stream (S1), Salado River at Esperanza Camping (S2) and Salado River at Santo Tomé Beach (S3).

Table 4
Integrated Biomarker Response (IBR) maximum, minimum and mean values for each site. ** Significant difference from the control group (AS), p<0.01.

IBR values	Minimum	Maximum	Mean	SD
AS	-0.04	0.24	0.02	0.11
S1	16.39	20.82	18.55**	2.37
S2	6.87	11.30	7.64	1.79
S3	11.35	15.95	13.19**	1.71

massive fish mortality event in the lower basin of Salado River, Lajmanovich et al. (2021) registered between Esperanza and Santo Tomé cities in sediments, glyphosate (20-60 µg/kg), and in fish tissues (*Prochilodus lineatus*) 2,4-D (20 µg/kg) and chlorpyrifos (30-80 µg/kg). Although most of these agrochemicals were detected in surface water samples, the highest concentrations, mainly glyphosate and AMPA, were detected in sediment samples. Related to these data, a north-south trend in the accumulation of glyphosate in lower Salado River sediments was inferred. In accordance with United States Environmental Protection Agency (Carey et al., 2008), the half-life of glyphosate in sediment is two orders of magnitude longer than in surface water. Previously, Ronco et al. (2016) reported for the first time glyphosate and AMPA in surface water and bottom sediments of main tributaries of the Paraná River basin, and at the confluence of the Salado and Carcaraña rivers. In these rivers, the authors found a high value (500 µg/kg) of glyphosate in sediment, which highlighted the great input of this pesticide into the Salado River lower basin.

Most commercial insect repellents are based on N,N-diethyl-m-methyl benzamide (DEET), as this compound is effective against a wide range of insects. Environmental DEET concentrations had been previously reported in basins of the south-east of Buenos Aires with a maximum value of 0.701 µg/L (De Gerónimo et al., 2014). In the present study, the concentration in sediment samples were higher in S1 and S2, which may implicate a higher risk of exposure for aquatic organisms. Due to its high-water solubility and moderate Koc (277), it can be easily transported by rainwater to surface water bodies near the application site. Despite that lethal and effective concentration of DEET informed in several aquatic species are above the environmental concentration found (Campos et al., 2016), up to our knowledge, there is no lethal and sublethal information of DEET toxicity on native amphibian species. Also, there is no previous data about the interaction between DEET, other pesticides and heavy metals simultaneously. For this reason, we propose to deepen this type of research.

The WQI showed a significant degradation of all sites. The WQI allowed to identify the physicochemical parameters considered in the calculation that were out of the limit for the protection of aquatic life by the Argentine Law 24.051, decree 831/93. However, the WQI did not allow to differentiate the sampling sites. These results highlight the need to analyze water quality with physicochemical parameters and ecotoxicological studies to provide ecologically relevant information.

In the present study, the IBR index allowed to integrate and quantify the sublethal effects of the sites and showed that larvae exposed to S1 followed by S3 were the most affected by the exposure to substances that caused oxidative stress, neurotoxicity and genotoxicity.

The complex matrix of water and sediment samples from S1 and S3 caused oxidative stress and a greater MN frequency on the exposed larvae. The enzyme CAT is one of the principal antioxidant enzymes in amphibians (Ferrari et al., 2009). An increase in the CAT activity indicates a higher formation of hydrogen peroxide, and an activation of the antioxidant defenses (Ferrari et al., 2011). Moreover, GST is a phase II detoxifying enzyme, which acts against contaminants and lipoperoxidation products by conjugating them with GSH (Ferrari et al., 2011). The increase observed in GST activity reflects an attempt to detoxify toxic compounds (Monferran et al., 2008). On the other hand, the increased GSH levels may be due of its synthesis as a consequence of the activation of the antioxidant defense system. GSH has an important role as a cofactor and substrate of several enzymes that maintain the redox status and acts itself as an antioxidant non-enzymatic defense (Ferrari et al., 2011). Oxidative damage to lipids (TBARS levels) was observed in larvae exposed to S1 and S3. These effects may be a consequence of the compounds detected in water and sediment samples, which are capable of inducing oxidative stress in amphibians, such as pesticides like glyphosate and atrazine (Dornelles and Oliveira, 2014), finopronil and 2,4-D (Freitas et al., 2022) and metals as As (Mardirosian et al., 2015), Cu and Cr (Huang et al., 2020). Moreover, the detected compounds may interact between them and with the physicochemical parameters and produce synergistic effects, such as is the case of glyphosate and As (Lajmanovich et al., 2019). In addition to oxidative damage to lipids, oxidative stress can affect other molecules such DNA. In this case, larvae exposed to S1 and S3, also presented a higher MN frequency, which may be a consequence of direct interaction of a xenobiotic with the DNA or by indirect causes such as oxidative stress. The observed effects may have deleterious effects for cells and tissues, which in the long term can comprise the survival of organisms (Fenech et al., 2020).

The complex matrix of water and sediment samples from all sites altered AChE activity. Also, the matrix samples from S1 and S2 altered BChE activity. In relation to substrates, BChE is less specific in terms of substrates. Thus, BChE has a more important role than AChE in the biotransformation. Also, BChE limits the number of cholinergic neurotoxins, such as organophosphorus insecticides, carbamate insecticides or even natural toxins (Carriquiriborde, 2021). The observed inhibition of BChE may indicate an attempt of organisms to detoxify neurotoxic compounds (Attademo et al., 2011). On the other hand, AChE activity was increased in larvae exposed to all sites. A possible consequence of the activation of AChE is a decrease in the acetylcholine (ACh) levels, which could be relevant due to the need for a precise level of ACh in the brain to preserve cognitive capacities (López et al., 2015). Some of the detected pesticides, such as metalaxyl, diazinon, 2,4-D and atrazine (Ahmed et al., 2011; da Fonseca et al., 2008; Xing et al., 2010), and/or metals such as Cd, Cu, Fe and Pb (de Lima et al., 2013), alone or in mixture, may alter the BChE and/or AChE activities. Alterations in the activity of these enzymes can lead to behavioral changes, which may negatively impact in populations and their viability (Sanchez-Hernandez, 2007).

In relation to lethal effect on *R. arenarum* larvae, only S1 produced significantly effects at chronic exposure times in accordance with the higher IBR calculated. The concentrations of metals and pesticides present in water and sediments from S1 were below the concentrations that cause lethal effects in *R. arenarum* larvae (Aronzon et al., 2011; Brodeur et al., 2009; Sztrum et al., 2011). However, the observed lethality can be explained by the high number of metals and pesticides detected and the interactions between them and physicochemical parameters.

4. Conclusions

A degradation of the studied sites was observed according to the obtained physicochemical and ecotoxicological information. So, exhaustive space-time monitoring regarding to the presence of agricultural and industrial contaminants are needed. As a first mitigation measure, there is an urgent need to increase the distance of pesticide-dependent GM crops from aquatic ecosystems. In conclusion, the observed degraded environmental quality of the Salado River basin threatens the social-cultural services, the human population and the environment.

CRediT authorship contribution statement

Julieta Peluso: Conceptualization, Data curation, Investigation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Carolina M. Aronzon:** Investigation, Funding acquisition, Resources, Supervision, Conceptualization, Methodology, Writing – review & editing. **Agostina Martínez Chehda:** Investigation, Formal analysis. **Ana Paula Cuzziol Boccioni:** Investigation, Formal analysis. **Paola M. Peltzer:** Conceptualization, Investigation, Visualization, Project administration, Resources. **Eduardo De Geronimo:** Methodology. **Virginia Aparicio:** Methodology. **Florencia Gonzalez:** Methodology. **Lautaro Valenzuela:** Methodology. **Rafael C. Lajmanovich:** Investigation, Formal analysis, Visualization, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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