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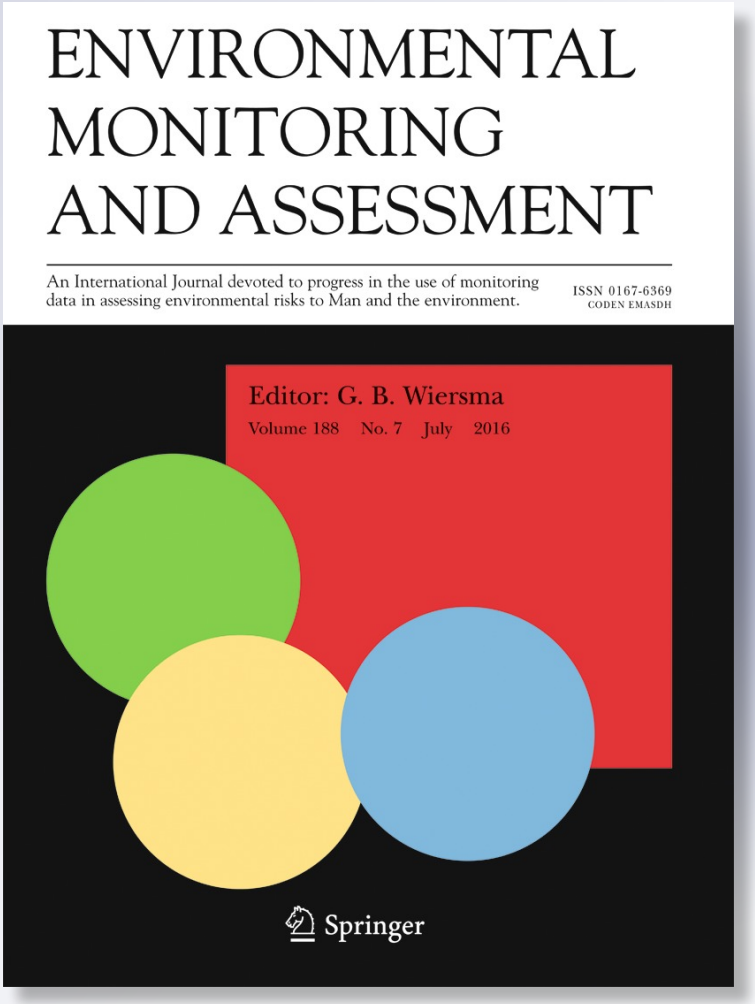

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Adverse effect of agroecosystem pond water on biological endpoints of common toad (*Rhinella arenarum*) tadpoles

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Abstract Chemical prroducts used in farming and wastes from livestock can contaminate pond water in agroecosystems due to runoff. Amphibians using these ponds for breeding are probably exposed to pollutants, and serious consequences might be observed afterward at the population level. Assessment biological endpoints of anuran to water quality give a realistic estimate of the probability of occurrence of adverse effects and provide an early warning signal. In this study, the ecotoxicity of agroecosystem ponds from the south of Córdoba province, Argentina, was investigated. Ponds in four sites with different degrees of human disturbance were selected: three agroecosystems (A1, A2, A3) and a site without crops or livestock (SM). The effect of pond water quality on the biological endpoint of *Rhinella arenarum* tadpoles was examined using microcosms with pond water from sites. Biological endpoints assessed were as follows: mortality, growth, development, morphological abnormalities (in body shape, gut, and labial tooth row formula), behavior, and blood cell parameters (micronucleus and nuclear abnormalities). Results indicated that water from agroecosystems has adverse effect on early life stage of *R. arenarum.* High mortality and fewer metamorphs were recorded in the A1 and A3 treatments. Tadpoles and metamorphs from A1 and A2 treatments had lower body condition. Tadpoles from A1 and A3 showed the highest prevalence of morphological abnormalities. The lowest amount of tadpoles feeding and the highest percentage of tadpoles swimming on the surface were observed in treatments with agroecosystem pond water. The higher frequencies of micronuclei and nuclear abnormalities were recorded in tadpoles from A1, A2, and A3 treatments. We check the sensitivity of the biological endpoints of *R. arenarum* tadpoles like early warning indicators of water quality. We found that the poor water quality of agroecosystem ponds has impact on the health of the tadpoles, and this could affect the persistence of populations. We recommend implementation of management actions before the harmful effects of agroecosystem pond water on early life stage of anuran become evident in higher ecological levels.

Keywords Anuran · Metamorphs · Mortality · Morphological abnormalities · Blood biomarkers · Agroecosystems

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Introduction

Agriculture covers approximately 38 % of the Earth's land surface, and it is vital to human sustenance (Foley et al. 2005, 2011; Ramankutty et al. 2008). However, its development is affecting wildlife in several ways (Donald 2004; Foley et al. 2005; Kareiva et al. 2007). Freshwater and terrestrial habitat loss and degradation as a result of anthropogenic activities are considered to be the primary and most pervasive threat to amphibian populations (Wake 1991; Hecnar and M'Closkey 1998). It has been estimated that an 89 % of the anuran species are affected by agriculture (GAA 2004; Young et al. 2004). Chemical products used in farming activities and the wastes of cattle can contaminate water bodies due to runoff and cause damages to aquatic environments (Knutson et al. 2004; Schmutzer et al. 2008). High concentrations of elements such as phosphorous and nitrogen have been detected in water bodies situated within agricultural areas (Hamer et al. 2004). These nutrients in high concentrations cause eutrophication of aquatic systems with a drastic decrease of dissolved oxygen and further death of all aerobic organisms (Mitsch and Gosselink 2000). In addition, contaminants such as metals and agrochemicals present in aquatic systems have lethal and sublethal effects on amphibians (Rowe et al. 2002; McDaniel et al. 2004; Metts et al. 2012).

Amphibians possess several characteristics that may make them more sensitive to environmental disturbances than other wildlife (Rowe et al. 2003; Hopkins 2007). The permeability of the skin and eggs makes them particularly sensitive to changes in hydric conditions as well as contaminants and certain skin diseases. Moreover, their aquatic–terrestrial life cycle places them in "double jeopardy" because a disturbance to the quality or availability of either habitat can disrupt their life cycle and affect populations (Dunson et al. 1992). Also, the high conversion efficiency of amphibians should also be associated with high rates of contaminant bioaccumulation compared with other animals of similar trophic position (Unrine et al. 2007). Anuran tadpoles are sensitive to alterations in their aquatic environment and are indicators of harmful levels of pollution (Cooke 1981). Water from deteriorated ponds in agroecosystems affects amphibian larvae, reducing its survival and affecting the biological endpoints (Marco and Blaustein 1999; Peltzer et al. 2008, 2013). Impacts on the early stages of development can negatively affect the recruitment of metamorphs (Schmutzer et al. 2008; Burton et al. 2009), so the populations and structure of the amphibian communities are affected (Gray et al. 2004). Thus, studies that focus on the effect of contaminants present in water bodies over the health of amphibian larvae provide crucial information for their conservation (Ficken and Byrne 2012) and constitute a warning sign of the level of modification of a given environment (Lajmanovich et al. 2010; Da Rocha 2011).

The South American common toad, *Rhinella arenarum* (Hensel 1867), is an anuran native species used as bioindicator organism, and the assessment of biomarkers on their larval stages is a suitable tool for biomonitoring of aquatic ecosystems (Venturino et al. 2003). Sensitivity of *R. arenarum* was proven in several studies (Howe et al. 1998; Vera Candioti et al. 2010; Bosch et al. 2011; Lajmanovich et al. 2014). This anuran species has a wide Neotropical distribution (Frost 2014), and it is commonly found in forests, wetlands, riversides, and urban and agricultural lands. Besides, this species is easy to handle and it acclimates well to laboratory conditions (Kwet et al. 2004).

The central region of Argentina has been greatly affected by the agricultural expansion (INTA, 2003; Rossi 2006), and as a consequence, many of the aquatic habitats in this area have been altered. Previous papers showed, in the sites studied in this research, that richness of amphibian communities was affected (Bionda et al. 2011, 2013). In addition, genotoxic damage and morphological abnormalities have been recorded in adults (Peltzer et al. 2011; Bionda et al. 2012; Caraffa et al. 2014) and in tadpoles (Babini et al. 2015a). The aim of this study was to evaluate the effect of agroecosystem pond water on early life stage of anuran using *R. arenarum* as the bioindicator species. The effect was assessed through the study of biological endpoints: mortality, growth, development, morphological abnormalities (in body shape, gut, and labial tooth row formula), behavior, and blood cell parameters (micronucleus and nuclear abnormalities).

Material and method

Study area and sampling sites

Ecotoxicity of different agroecosystem ponds from the south of Córdoba province, Argentina (33° 10′

S–64° 20′ O; 420 meters above sea level (m a.s.l.)), on early life stage of anuran was investigated. The main socioeconomic activities of this area are farming and livestock. Agriculture is intensive, and it requires the application of pesticides and fertilizers in great amounts (CASAFE 1999; Lajmanovich et al. 2005a). Over 80 % of forests have been lost in the last 20 years with a deforestation rate 12 times higher than the world average (Nori et al. 2013).

We selected ponds in four sites with different degrees of human disturbance. Three sites corresponded to the following agroecosystems: *A1* (crop and cattle, 33° 05′ 51″ S, 64° 26′ 02″ O; 471 m a.s.l.), *A2* (crop, 33° 06′ 09″ S, 64° 25′ 32″ O, 467 m a.s.l.), and *A3* (crop and cattle, 33° 05′ 39″ S, 64° 25′ 58″ O, 468 m a.s.l.). The other site is in a protected natural area (the native forest "El Espinal," located within the National University of Río Cuarto) and is not affected by agriculture or cattle: SM (33° 06′ 42 S, 64° 18′ 12 O, 428 m a.s.l.). Native species such as *R. arenarum* are known to use these ponds for reproduction, and unhealthy tadpoles were found in these sites (Babini et al. 2015a, b).

Experimental design: microcosms

The effects of pond water on biological endpoints were examined in outdoor microcosms. The microcosm experiment is based on a modification of the methodology proposed by Peltzer et al. (2013). Egg samples of three distinct zones (each approximately 50 g) of the same clutch (363 g, about 40.900 eggs) from A3 were extracted for the husbandry of tadpoles in the lab. This reduced the genetic effect because all the eggs come from the same parents. The eggs were placed in an aquarium (transparent container) with dechlorinated water. The aquarium was close to a natural light source. In order to generate movement of the water, a submersible water pump was placed inside the aquarium. To avoid the suction of the eggs, the water pump was covered with a micronet. When hatchlings reached stage 23 (free-swimming hatchlings: period of transition from a rather immobile embryo to an actively feeding tadpole, atrophying external gills, and the spiracle formed Gosner 1960), they were transferred from the aquarium to the microcosms (experimental day 0) to complete their development.

Microcosms consisted of plastic enclosures (37 cm long × 25 cm wide × 20 cm deep) placed outdoors and covered with a mesh to avoid oviposition by insects or predation by birds. Each microcosm had 15 L of pond water from one of the sites studied. Water volume was kept constant. Each microcosm was stocked with 20 hatchlings, resulting in a density, which is within the range of densities found in nature (14–4238 hatchlings/1000 L; Werner and Glennemeier 1999). Tadpoles were fed ad libitum with boiled *Taraxacum* sp. Water of microcosms was renewed weekly with water from the corresponding ponds in the study sites. A total of 12 microcosms were set (three for each site). All of them were totally drained on day 45, when ponds in locations A1 and A2 were dried.

Biological endpoints

After the beginning of the experiment (day 1), microcosms were checked daily and the presence of dead tadpoles or metamorphs was registered. Individuals found dead were fixed in a phosphate buffer for analysis. Metamorphs in stages 44–45 were anesthetized with a solution of MS 222 or methanesulfonate salt (3-aminobenzoic acid ethyl ester, Sigma-Aldrich™) at 0.5 % and preserved in phosphate buffer for further analysis. Individuals were considered to be metamorphs from stage 42 (Gosner 1960) when tadpoles have lost its larval characteristics and acquired adult structures, i.e., emergence of at least one forelimb (Peltzer et al. 2013).

At 20 days of the beginning of the experiment, six tadpoles from each treatment were collected by random sweeps with a net to perform morphometric analysis and check for morphological abnormalities and blood biomarkers. On day 45, the remaining tadpoles (larvae alive still without metamorphosed) were anesthetized and fixed for morphometric analysis and morphological abnormalities. Morphometric parameters measured on tadpoles and metamorphs were as follows: development stage (following Gosner 1960), mass by means of a Mettler balance (P11N 0–1000 g), and snout–vent length (SVL) with a Zeiss West Germany binocular magnifying glass. All individuals found dead were also included in the morphological abnormality analysis. Variations in phenotype were classified as morphological abnormalities following Krishnamurthy and Smith (2011), Peltzer et al. (2013), and Babini et al. (2015a). Each preserved specimen was objectively scored for the following: (a) body shape (swollen body: bulging of the larvae body and with sac appearance or diamond shape: emaciated body surface and lack of a smooth oval contour), (b) intestine (normal or with abnormalities: gut uncoiling or

diverted gut), and (c) labial tooth row formula (LTRF) (normal: 2(2)/3, 2(2)/3(1) or abnormal: without labial teeth). The prevalence of a certain type of abnormality was calculated by dividing the number of larvae with this abnormality by the number of individuals examined. The variations in phenotype were registered with a Zeiss West Germany binocular magnifying glass.

Behavioral changes in response to perturbations in aquatic environment have been noted like disrupting foraging or fast and erratic swimming behavior in tadpoles (Newcombe and MacDonald 1991; Rowe et al. 1996; Krishnamurthy and Smith 2011; Wood and Richardson 2009). Studying the behavior of tadpoles was based on a modification of the methodology of Wood and Richardson (2009) and Krishnamurthy and Smith (2011). Activity of tadpoles was recorded once a week. For a period of 2 min, we counted the number of tadpoles feeding or swimming near surface in each microcosm. These data were expressed as a percentage per treatment according to the total number of larvae in each microcosm at that time.

To analyze blood biomarkers, six tadpoles from each treatment (collected at 20 days of the beginning of the experiment) were anesthetized with a solution of MS 222 at 0.5 %. Blood was obtained by cardiac puncture (Lajmanovich et al. 2005b), and blood smears were prepared on clean slides, fixed, and stained according to the May–Grünwald/Giemsa method (Dacie and Lewis 1995; Barni et al. 2007). Coded and randomized slides were scored by a single observer using a Carl Zeiss trinocular Primo Star microscope (Pack 5) with ×100 objective lens and immersion oil (Feng et al. 2004). It is important to note that red blood cells in amphibians are nucleated and undergo cell division in the circulation, particularly during the development stages (Duellman and Trueb 1986). Criteria for distinguishing a micronucleus (Mn) are as follows: (a) the intensity of a stained Mn should be similar to that of the principal nucleus but with a smaller diameter, (b) it should be round with a nuclear membrane and not connected to the principal nucleus, and (c) it should not overlap with the principal nucleus and has to be located within the cytoplasm (Schmid 1975; Ferrier et al. 1998; Fenech 2000; Meintières et al. 2001).

Presence of other nuclear abnormalities (NA) was assessed according to the procedures of Guilherme et al. (2008), Pollo et al. (2012), and Lajmanovich et al. (2014) by examining mature erythrocytes and determining the frequency of the following nuclear lesions: lobed nuclei (*L*), multinucleate or segmented nuclei (*S*), kidney-shaped nuclei (*K*), notched nuclei (NN), and pyknotic nuclei (PN). The sum of all lesions observed ($L + S + K + NN + PN$) was expressed as NA frequency. The frequency of mitotic and enucleated erythrocytes (erythroplastids) was also recorded. The frequencies of Mn and NA were determined in 2000 erythrocytes from each tadpole, and the results were expressed per 1000 cells (‰).

Water parameters

Water pH, temperature, conductivity, total dissolved solids (TDS), and salinity were measured with a multiparameter test (35-series 35425-10, Oakton Instruments 625 E Bunker Court Vernon Hills, IL 60061, USA), and transparency of water was estimated with a transparency tube. Water parameters were measured on each microcosm after each water exchange.

Water chemical analyses were performed on water samples. These water samples were collected at the same time that the water was collected for filling microcosms. Analyses were performed by the area of Hydrology, Department of Geology, National University of Río Cuarto, and these were performed according to the standard methods of APHA-AWWA-WEF (1998). Carbonates and bicarbonates were measured by potentiometric titration with Thermo Orion-selective electrode, sulfates were measured by turbidimetry (with centrifugal Macrotronic), chloride was measured by colorimetric titration with silver nitrate, calcium and magnesium were measured by colorimetric titration with EDTA, and sodium and potassium were measured by flame photometry (315 Metrolab digital photometer) and fluoride by ion-selective electrode (Orion–Thermo). Nitrates are determined by potentiometry with ion-selective electrode (Orion Model 9307), reference electrode, and Orion potentiometer 710A. To calibrate the potentiometer, six benchmarks (5, 10, 25, 50, 100, and 300 mg/L NO_3^-) were used. The detection limit of the determination of NO_3^- is 0.2 mg/L, and the analytical error of the measurements is 0.5 %.

Statistical analysis

Normality and homogeneity of variance were assessed by Kolmogorov–Smirnov and Levene tests, respectively, using different software packages according to the

analysis: InfoStat/P version 1.1 (Di Rienzo et al. 2012) and Statistica (StatSoft 2001).

Water parameters were analyzed by ANOVA block design. We found that the differences between the treatments are greater than the differences between the microcosms of equal treatment (pH: $F_{2,48} = 0.38$; $p = 0.6865$; conductivity: $F_{2,48} = 0.62$; $p = 0.5422$; salinity: $F_{2,48} = 0.63$; $p = 0.5374$; TDS: $F_{2,48} = 0.61$; $p = 0.5481$; transparency: $F_{2,48} = 0.44$; $p = 0.6468$). As we did not record statistical differences in water variables among microcosms of equal treatment and as we did not record statistical differences in tadpole mortality (Fisher's exact probability test $p < 0.05$), this data was pooled per treatment following Peltzer et al. (2013).

Water parameters were compared between treatments by ANOVA. Differences in the proportion of mortality, metamorphs, and type of abnormality between treatments were examined with the binomial test at $p < 0.05$ significance. Mass (*M*) and snout–vent length (SVL) were log-transformed. Measures of tadpoles and metamorph *M* were regressed on SVL, and the residuals were taken as an index of body condition (Wood and Richardson 2009). Then, body conditions of tadpoles and metamorphs between treatments were analyzed by ANOVA. N lesions in tadpoles' erythrocytes were analyzed with ANOVA. In all cases, the Di Rienzo, Guzmán and Casanoves (DGC) post hoc test was used (Di Rienzo et al. 2002). This test uses the multivariate technique of cluster analysis (average chain or UPGMA) on a distance matrix obtained from the sample means (Balzarini et al. 2008).

Canonical correspondence analysis between water parameters and biological endpoints of tadpoles was performed. The analysis was performed with Canoco for Windows 4.5 program, and the TRIPLOT was performed with Canodraw for Windows program (ter Braak and Smilauer 2002).

Results

Water parameters

The water parameters from the different sites studied are shown in Table 1. Phosphate values were higher in agroecosystem ponds, nitrate was detected only in A1 and A2 sites, and fluoride was higher in A3 that in the other ponds. The values of water variables measured in treatments are shown in Table 2. ANOVA showed significant differences in water variables between treatments: (pH: $F_{3,56}$ 11.51, $p < 0.0001$, DGC test: *df* 56; PCALT 0.21) (conductivity: $F_{3,56}$ 23.65, $p < 0.0001$, DGC test: *df* 56; PCALT 85.79) (salinity: $F_{3,56}$ 23.21, $p < 0.0001$, DGC test: *df* 56, PCALT 42.39) (TDS: $F_{3,56}$ 23.72; $p < 0.0001$, DGC test: *df* 56; PCALT = 60.94) (transparency: $F_{3,56}$ 139.97, $p < 0.0001$, DGC test: *df* 56; PCALT = 0.39). SM and A2 had the lowest mean values of conductivity, TDS, and salinity. A3 showed more basic pH values. Treatments with water from agroecosystems showed lower transparency values while in treatment with water from SM site (Table 2).

Growth, mortality, and development

At 20 days, there was no significant difference in body condition (BC) of tadpoles between treatments. However, after 45 days of the start of the experiment, there was a significant difference in BC of remaining tadpoles (alive without metamorphosed) between SM and A1 (ANOVA: $F_{3,13}$ 3.47; $p = 0.0478$; DGC test: *df* 13; PCALT = 0.0153). Metamorphs from SM treatment had better BC, and it was significantly different from

Table 1 Water chemical analysis summary

	Ponds			
	A1	A2	A3	SM
Color	Amber	Dark amber	Amber	Amber light
pH	7.3	6.9	8.6	7.7
Carbonate	0	0	21.8	0
Bicarbonate	182.5	157.5	555	320
Sulfate	44.4	50.8	89.8	34.2
Chloride	7.1	7.1	128.6	28.6
Sodium	21.2	30.3	186	37.4
Potassium	28.4	38.7	80.9	22.6
Calcium	24	10.4	48	60.8
Magnesium	13.7	8.3	12.2	5.4
Nitrate	1	1	ND	ND
Nitrite	ND	ND	ND	ND
Fluoride	0.4	0.4	2	0.3
Phosphate	9	9	9	5.7

Ion concentrations are shown in milligram per liter. The detection limit of the determination of NO_3^- is 0.2 mg L^{-1}, and the analytical error of the measurements is 0.5 %

ND not detected or observed; *A1*, *A2*, *A3* ponds in agroecosystems; *SM* pond in natural area

Table 2 Water parameters of different treatments on the experimental period

	Treatments			
	A1	A2	A3	SM
Conductivity (μS/cm)	503.5 ± 80.4	278.7 ± 42.4	584.3 ± 198.2	341.3 ± 56
	B	A	B	A
Salinity (mg L^{-1})	243.6 ± 39.5	134.5 ± 19.9	284.3 ± 98.4	164.7 ± 27.1
	B	A	B	A
TDS (mg L^{-1})	357.1 ± 57	197.5 ± 30	414.7 ± 140.8	241.4 ± 40.1
	B	A	B	A
pH	8 ± 0.3	7.75 ± 0.2	8.32 ± 0.2	8.13 ± 0.4
	B	A	C	B
Transparency (cm)	16.7 ± 0.4	16.8 ± 0.3	16.6 ± 0.4	19.8 ± 0.9
	A	A	A	B

Data are shown as mean ± standard deviation. DGC TEST: means of a row with the same letter are not significantly different ($p > 0.05$)
A1, *A2*, *A3* treatments with water of agroecosystems; *SM* treatment with pond water in natural area

that of A1 and A2 treatments (ANOVA: $F_{3,44}$ 3.54; $p = 0.0222$; DGC test: *df* 44; PCALT = 0.56).

At day 20, the highest mortality was recorded in A3 (52 %), but at day 45, by the end of the experiment, the highest mortality was observed in A1 (73 %). The proportion of mortality was lower in SM, and it was significantly different from that of A1 ($Z_{SM\text{-}A1}$ −0.2, $p = 0.0249$) (Table 3).

At day 20, the greatest amount of metamorphs was recorded in A1 (12 %). But at day 45, the greatest amount of metamorphs was observed in SM (39 %) and it was significantly different from that of A1 ($Z_{A1\text{-}SM}$ −0.18, $p = 0.0321$). The mean number of days until complete metamorphosis was lower in A1 treatment. Individuals needed a minimum of 25 days from the start of the experiment (minimum value recorded in A1) to complete metamorphosis, i.e., 43 days from the collection of clutch until metamorphosis (Table 3).

Behavior

The highest percentage of larvae swimming near the surface was recorded in the agroecosystem water treatments, and there was a significant difference between SM and A2 ($Z_{SM\text{-}A2}$ −0.32, $p < 0.0001$) and A3 ($Z_{SM\text{-}A3}$ −0.24, $p = 0.0004$). The lowest amount of larvae feeding was recorded in A1 and A3, and there was a significant difference between SM and A3 ($Z_{SM\text{-}A3}$ 0.14, $p = 0.0141$) (Table 3).

Morphological abnormalities

The three types of abnormalities were observed in all sites (Table 3). The highest prevalence of abnormalities in LTRF and intestine was recorded in A1, while the highest prevalence of inverted diamond shape was recorded in A3. There were differences in the abnormalities total registered between SM and A3 ($Z_{SM\text{-}A3}$ −0.4, $p = 0.0128$).

Blood cell parameters

Different nuclear lesions were observed (Table 4). Tadpoles from treatments with water from agroecosystems showed high frequencies of micronuclei or abnormal nuclei. Statistical analysis showed significant differences in the frequency of Mn between A1, A2, and A3 compared to SM (ANOVA: $F_{3,21}$ 3.40; $p = 0.039$; test DGC: *df* 21; PCALT = 1.0). There were also significant differences in the frequencies of NA between A1, A2, and A3 compared to SM (ANOVA: $F_{3,21}$ 7.42; $p = 0.0017$; test DGC: *df* 21; PCALT = 14.2). Enucleated erythrocytes were only observed in tadpoles from A1, A2, and A3.

Water parameters and biological endpoints

Canonical correspondence analysis (CCA) indicated that the accumulated inertia of ratio between water

Table 3 Mortality, development, behavior, and morphological abnormalities of *Rhinella arenarum* in different treatments on the experimental period

	Treatments			
	A1	A2	A3	SM
Mortality				
At 20 days (%)	41.7	18.3	51.7	41.7
At 45 days (%)	73.3	55	68.3	53.3
Metamorphosis				
At 20 days (%)	11.7	6.7	3.3	5
At 45 days (%)	21.7	36.7	25	39
Days to reach metamorphosis (from stage 23)	27 ± 2	38 ± 2	41 ± 2	40 ± 2
Behavior				
Swimming near surface (%)	62	86	78	54
Feeding (%)	19	31	13	27
Abnormalities in				
LTRF (prevalence)	0.286	0.091	0.111	0.077
Body shape (prevalence)	0.143	0.182	0.444	0.154
Gut (prevalence)	0.286	0.182	0.222	0.154
Total abnormalities (prevalence)	0.714	0.454	0.777	0.385

For each treatment, 60 tadpoles underwent in total

A1, *A2*, *A3* treatments with water of agroecosystems; *SM* treatment with pond water in natural area

parameters and biological endpoints of tadpoles in the first axis was 68 %. The first factorial plane collects 89 % of the total inertia of CCA, well sufficient to summarize the information on the relationship between water parameters and the biological endpoints. Collinearity was detected between conductivity, salinity, and TDS, so salinity and TDS were removed from the analysis.

The TRIPLOT (Fig. 1) shows that the first axis separates the SM treatment of the A1, A2, and A3 treatments. Transparency, calcium, and water hardness had an inverse relation to the rest of the water parameters. SM treatment had relation to transparency, and it was associated with the biological endpoints: BC of metamorphs, metamorphosis, and feeding behavior. The A1 and A3 treatments had relation to conductivity,

Table 4 Blood cell parameters (in frequency thousand, ‰) of *Rhinella arenarum* tadpoles in different treatments

	Treatments			
	A1	A2	A3	SM
Micronuclei	5.73 ± 1.74	3.41 ± 1.18	6.24 ± 1.79	1.61 ± 0.7
Nuclear Abnormalities	42.47 ± 4.79	53.23 ± 3.58	53.05 ± 5.78	26.56 ± 3.64
Notched	11.03 ± 2.17	11.6 ± 1.93	13.58 ± 1.99	6.85 ± 0.93
Kidney-shaped nuclei	5.04 ± 0.9	8.47 ± 1.75	5.84 ± 0.75	6.24 ± 1.24
Lobed nuclei	25.93 ± 3.61	31.46 ± 3.08	31.89 ± 4	12.85 ± 2.46
Multinucleated cell	0.47 ± 0.33	1.7 ± 0.55	1.74 ± 0.39	0.63 ± 0.31
Mitotic erythrocytes	1.09 ± 0.56	2.27 ± 0.49	1.43 ± 0.6	0.16 ± 0.16
Enucleated erythrocytes	0.94 ± 0.35	0.38 ± 0.24	1.39 ± 0.7	ND

Data are shown as mean ± standard deviation

A1, *A2*, *A3* treatments with water of agroecosystems; *SM* treatment with pond water in natural area, *ND* not detected or observed

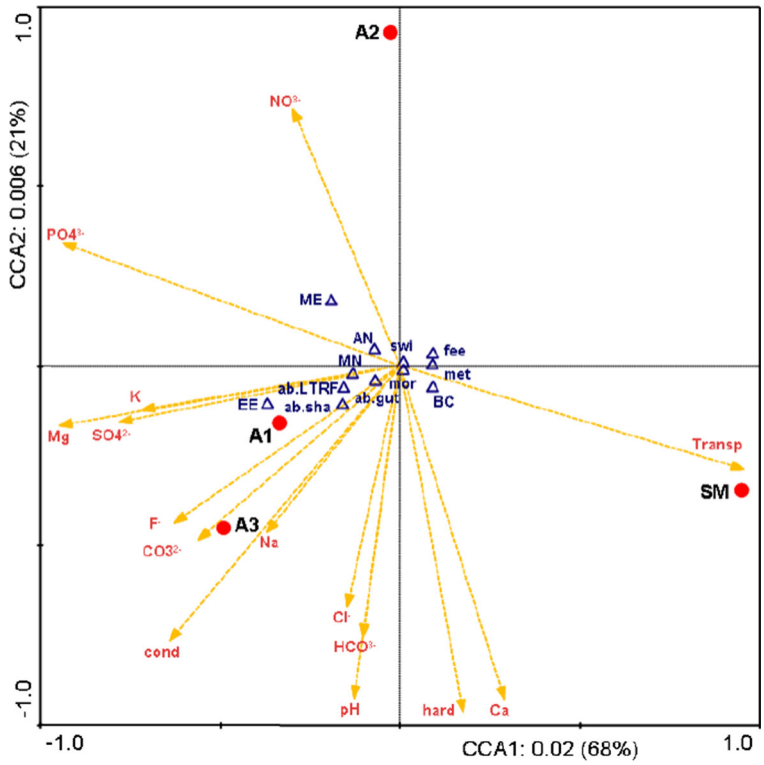

Fig. 1 TRIPLOT (first two CCA axes) showing the treatments (*circles*), the biological endpoints (*triangles*): *mor* mortality, *met* metamorphosis, *ab.LTRF* abnormal LTRF, *ab.sha* abnormal body shape, *ab.gut* abnormal gut, *swi* behavior swimming near surface, *fee* behavior feeding, *MN* micronuclei, *ME* mitotic erythrocytes, *EE* enucleated erythrocytes, *AN* nuclear abnormalities and the water parameters (*arrows* represent the correlation of the physical variables with the canonical axes): CO_3^{2-} carbonate, HCO^{3-} Bicarbonate, SO_4^{2-} sulfate, Cl^- chloride, *Na* sodium, *K* potassium, *Ca* calcium, *Mg* magnesium, NO_3^- nitrate, F^- fluoride, $PO4^{3-}$ phosphate, *cond* conductivity, pH, *hard* water hardness, *transp* transparency

carbonate, fluoride, sodium, sulfate, magnesium, and potassium, and these were associated with enucleated erythrocytes, abnormal body shape, abnormal LTRF, abnormal gut, and micronuclei. A2 treatment had relation to nitrate and phosphate, and it was associated with mitotic erythrocytes and NA.

Discussion

Cellular (cellular and NA) and organismal (growth, behavior, survival, abnormalities) endpoints are early warning indicators to unacceptable environmental conditions because many of the responses at these levels occur within a short time of exposure to a stressor and thus allow for relatively rapid determinations of environmental conditions (Hawkins 2007). Ponds located in agroecosystems receive washed-off contributions from the surrounding farmland, such as nutrients and pesticides. Amphibians that use these ponds for breeding are probably exposed to water pollutants, and some serious consequences may then be observed at the population level (Vonesh and de la Cruz 2002). The analysis of tadpoles' biological endpoints exposed to pond water in microcosms indicated that agroecosystem water might be harmful for early life stages of *R. arenarum.*

Mortality, growth, and development

High mortality and fewer metamorphs were recorded in the A1 and A3 treatments. Tadpoles and metamorphs from A1 and A2 treatments had lower BC. A high mortality was also registered by Peltzer et al. (2008) in tadpoles of agroecosystem ponds. They reported that the physical–chemical variables that contributed significantly to variability of anuran survival were nitrate and orthophosphate concentrations. We registered higher levels of nitrate and phosphate in ponds of the three agroecosystems studied compared to SM site. These are the main components of agrochemicals and are good indicators of the existence of disturbance by agriculture and livestock (Hamer et al. 2004; Giuliano and Blarasin 2013). Nitrate concentration recorded in A1 and A2 is the maximum value of the range (0.5–1.0 mg/L) suggested by Camargo and Alonso (2007) to prevent the processes of acidification and eutrophication of aquatic ecosystems and protect aquatic animals (and people) from the toxic effects of nitrogen compounds. Nitrate exerts a toxic action in organisms, because in presence

of nitrate, the respiratory pigments are converted into incompetent forms not able to transport or release oxygen (Camargo and Alonso 2007). Amphibians, especially in the larval stage, are very sensitive to nitrate, even in low concentrations. Several studies indicate that widespread use of nitrogenous fertilizers ($NH4NO_3$, KNO_3, $NaNO_3$) could be contributing significantly to the decline of amphibian populations in many areas of the world (Marco 2002). Besides, phosphate and nitrate favor the proliferation of primary producers and then their death and decay, leading to a drastic decrease in the concentration of dissolved oxygen in ecosystems with low water renewal rate. All aerobic organisms in the community, including aquatic stages of amphibians, are adversely affected by this reduction of oxygen (Mitsch and Gosselink 2000). Nitrite was not detected in any of the studied sites, possibly because they are rapidly oxidized to nitrate, resulting in only trace levels usually found in surface waters (Russo 1985).

Fluoride may have also affected tadpoles. Concentration of fluoride in A3 (2 mg/L) exceeded the reference level (1.4 mg/L) proposed by Argentinean government agencies (Law 24051 1993), and all sites displayed concentrations of fluoride over the limit suggested (0.12 mg/L) by the Canadian Council of Ministers of Environment (CCME 2003) for the protection of the aquatic biota. In uncontaminated freshwater ecosystems, the concentration of fluoride varies from 0.01 to 0.3 mg/L (Camargo 2003), but these levels can be increased by human activities. Significant increases are caused by the use of pesticides containing fluoride (Camargo 2003). Fluoride ions act as enzymatic poisons, inhibiting enzyme activity and, ultimately, interrupting metabolic processes such as glycolysis and synthesis of proteins. Its toxicity increases with increasing concentration, exposure time, and water temperature and decreases with increasing body size and calcium and chloride concentrations. Moreover, high concentrations of sodium can increase the solubility of fluoride (Camargo 2003) and we detected that water from A3 had the highest concentration of sodium.

Toxicity of water pollutants may change depending on physical and chemical characteristics of water (CCME, 2003). A1–A3 and A2–SM treatments had similar conductivity, salinity, and TDS. Transparency was always lower in those treatments with agroecosystem water. Transparency allows to infer water clarity and limitation of light penetration, which in turn can reduce photosynthesis (CCME, 2003). Transparency relates inversely to turbidity, water color, total solids, and demand for chemical oxygen, and it relates directly to pH and concentration of dissolved oxygen (Betanzos Vega et al. 2013).

Wood and Richardson (2009) reported tadpoles from treatments with lower transparency because receiving sediment additions showed reduced survival to metamorphosis, although no treatment effects were detected on time to metamorphosis. This is consistent with our results of lower survival levels in tadpoles bred in lower transparency waters. But, we did find differences in time to metamorphosis. Time to reach metamorphosis was lower in A1 treatment than in the other treatments. Maybe, this is because tadpoles are forced to enter metamorphosis immediately after reaching the minimum critical size in order to escape the poor aquatic conditions (Wood and Richardson 2009).

Abnormalities

Tadpoles from A1 and A3 showed high prevalence for the studied morphological abnormalities. Krishnamurthy and Smith (2011) reported that organophosphate pesticides widely used in agriculture can increase the frequency of body shape abnormalities in tadpoles. Likewise, Peltzer et al. (2013) reported that the nutrient-rich sediments of a soybean field pond could be responsible for the higher prevalence of diverted and inverted guts. Furthermore, Babini et al. (2015a) found a high frequency of abnormal LTRF and tadpoles without labial teeth in agroecosystem ponds.

Our results suggest that the appearance of abnormalities in tadpoles could be an indication of intoxication, and this may be useful as an assessment tool or indicator of contamination in agricultural ponds. Unfortunately, it seems that these abnormalities are followed by mortality, and thus, early detection may not help to save the tadpoles but may serve as an ecological indicator for water quality (Krishnamurthy and Smith 2011).

Behavior

Warner et al. (1966) were among the first to recognize the utility of behavioral measures since they are more sensitive than other biological endpoints. Abnormal behavior is one of the most conspicuous endpoints, but until recently, it has been underused by ecotoxicologists (Dell'Omo 2002). Behavioral measures have great potential as bioindicators of health status of organism,

because behavior is the physical manifestation of an animal's integrated physiological response to its environment (Clotfelter et al. 2004). The lowest amount of tadpoles feeding and the highest percentage of tadpoles swimming on the surface were observed in treatments with agroecosystem pond water. Rowe et al. (1996) reported a reduced foraging efficiency of tadpoles exposed to heavy metals, and Krishnamurthy and Smith (2011) reported that tadpoles in treatments with organophosphate pesticide showed less active feeding than tadpoles in the control treatment. Furthermore, Wood and Richardson (2009) reported disrupted foraging and erratic swimming behavior in tadpoles under turbid conditions. This is consistent with our results in low transparency water coming from agroecosystem ponds. In nature, such responses could expose tadpoles to increased predation pressure as they become more active, and there is likely an extra energetic cost to such behavioral responses (Wood and Richardson 2009).

Although there are few toxicology papers that include behavioral measures in amphibians, possibly, the behavioral differences observed between treatments are denoting the effect of water quality on larvae, since an animal's behavioral integrity is also an indicator of its health (Clotfelter et al. 2004).

Blood cell parameters

Amphibian hematological parameters may be affected by environmental stressors (Ilizaliturri-Hernandez et al. 2013). Erythrocytes have high incidence of Mn and NA after the exposure to various contaminants (Campana et al. 2003; Lajmanovich et al. 2005b; Cabagna et al. 2006). In a previous work (Babini et al. 2015a), we calculate the frequencies of Mn and NA for *R. arenarum* under laboratory conditions that could be considered as baseline frequencies. The Mn frequency was 0.20 ± 0.27, and NA frequency was 1.90 ± 1.29. These values are lower than the values that were recorded in all treatments. The higher frequencies of micronuclei were recorded in tadpoles from A1 and A3 treatments. These values may indicate a genotoxic effect and are in agreement with other studies, which found higher erythrocyte MN frequencies in tadpoles inhabiting agricultural ponds (Peltzer et al. 2008; Babini et al. 2015a). In the same way, these observations support the genotoxicity and/or cytotoxicity hypothesis as a consequence of the effects of chemicals that are used in farming (Gauthier et al. 1993; Lajmanovich et al. 2014).

We also observed an increment in the number of NA in treatments with water from agricultural ponds. Cell division is an essential condition for MN formation, and while little is known about mitotic activity in tadpoles, the stress could be affecting cell division rates (Lajmanovich et al. 2014), and this could represent a short-term means of increasing the oxygen-carrying capacity of the blood in amphibian species (Barni et al. 1995, 2002, 2007). Likewise, an increment in erythrocyte unusual forms, like lobed nuclei and segmented cytoplasm, has been reported earlier in situations of stress (Fijan 2002), and they are considered to be an expression of direct cell division. We also recorded a high frequency of mitotic erythrocytes in tadpoles from treatments with agroecosystem water. The presence of enucleated erythrocytes in agricultural ponds has also been related to stress and optimization of oxygen uptake (Barni et al. 2007) by improving the cell surface/volume ratio (Lajmanovichi et al. 2014) particularly in conditions of water pollution.

Conclusions and implications

Those factors which compromise larval and juvenile stages may affect population persistence (Vonesh and de la Cruz 2002). Biological endpoints of the anuran tadpoles give a realistic estimate of the probability of occurrence of adverse effects, providing an early warning signal. This experiment with microcosms allowed us to assess the effect of pond water on larvae of *R. arenarum*. The agroecosystem pond water treatments had higher mortality of tadpoles, fewer metamorphs, and lowest amount of tadpoles feeding. The tadpoles from these treatments had lower BC, increased prevalence of abnormalities in the morphology, and higher frequency of MN and AN.

Of the three agro-water treatments, the A1 and A3 treatments had a greater negative effect on mortality, metamorphosis, morphological total abnormalities, and micronuclei than A2 treatment. It should be noted that the A1 and A3 agroecosystems had crops and livestock, while the A2 agroecosystem had only crops. This is consistent with previous studies that have reported negative effects of cattle on some amphibian species (Burton et al. 2009; Jofré et al. 2007; Schmutzer et al. 2008; Bionda et al. 2011). Therefore, we recommend agricultural practices with less access of cattle to wetlands.

We check the sensitivity of the biological endpoint of *R. arenarum* tadpoles like early indicators of water quality and recommend implementation of management actions before the harmful effects of agroecosystem pond water on early life stage of anuran become evident in higher ecological levels.

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