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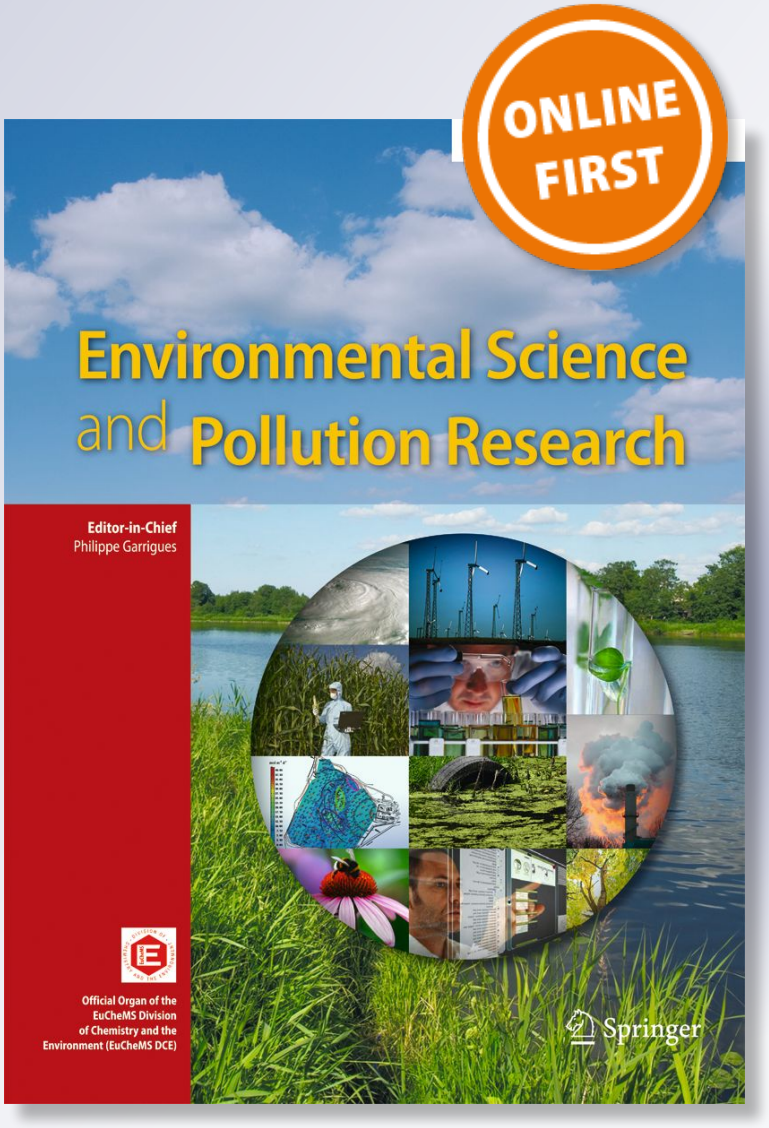

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RESEARCH ARTICLE

Evaluation in situ of genotoxicity and stress in South American common toad *Rhinella arenarum* in environments related to fluorite mine

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Abstract Little attention has been paid to the impact of wastewater generated by mining activities on fluoride. In this study, we evaluated the hematology responses of common South American toad *Rhinella arenarum* inhabiting natural and artificial environments associated with a fluorite mine from central Argentina. We analyzed three sampling stations associated with the fluorite mine: (I) Los Cerros Negros stream (CN), which runs on granitic rock with a high fluorite content; (II) Los Vallecitos stream (LV), which runs on metamorphic rock with low fluorite content; and (III) artificial decantation ponds (DP) containing sediments produced by fluorite flotation process. We calculated frequencies of micronuclei, erythrocyte nuclear abnormalities, mitosis, and immature erythrocytes. In addition, we performed a differential leukocyte count and determined neutrophils/lymphocyte ratio as a stress response estimator. We found high micronucleus (MN) and erythrocyte nuclear abnormality (ENA) frequencies in DP and CN but low frequencies in LV. The neutrophil/lymphocyte ratio was different among sites, with a significant increase in individuals from DP. Values registered in DP could be caused by exposure to mixture of compounds registered in dams that hold wastewater, while high values registered in CN stream might be due to natural concentrations of fluoride. Our results suggest that blood is an effective and non-destructive sensitive indicator for monitoring genotoxic agents in freshwater ecosystems.

Keywords Fluorite mine · Micronuclei · Nuclear abnormalities · Leukocyte · *Rhinella arenarum*

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Introduction

Wastewater generated by mining activities contains complex mixtures of assorted contaminants (Marques et al. 2009; Zocche et al. 2013; Lanctôt et al. 2016), raising concerns about possible threats to aquatic biota. Aquatic animals as amphibians could choose to exploit wetland habitats created by wastewater holding dams, presenting an additional possible exposure scenario (Lanctôt et al. 2016). Particularly, amphibians are sensitive to environmental changes because they have a highly permeable skin, which can easily absorb substances from the environment, and have a life cycle with aquatic and terrestrial stages (Young et al. 2004). These organisms have been used for biomonitoring studies because they respond rapidly to ecosystem changes. This vulnerability has also been responsible for decline in amphibian populations worldwide (Stuart et al. 2004).

Laboratory toxicity testing does not always generate ecologically relevant information because field situations may not be accurately simulated under laboratory conditions, and sample collection, storage, or handling can affect toxicity (Castro et al. 2003). In situ bioassays are an effective tool to overcome this problem, providing a more realistic approach, because these studies contemplate environmental phenomena such as bioavailability of compounds present in the environment and allow the interaction of multiple factors that are not often included in laboratory tests (Djomo et al. 2000; Castro et al. 2003; Antunes et al. 2008; Lavoie et al. 2012). Among the

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techniques for the detection of genotoxic effects, micronucleus (MN) test and erythrocyte nuclear abnormalities (ENA) are the most popular in ecotoxicology (Ayllon and Garcia Vazquez 2000; da Silva Souza and Fontanetti 2006; Machado da Rocha 2011) because of their simplicity, sensitivity, and sublethal detection of alterations which lead to early prevention and/or remediation (Livingstone 1993).

Based on our review of literature, the only study exploring in situ the genotoxicity in aquatic animals inhabiting wetland related to fluorite mining has been carried by Pollo et al. (2016), although in this work they were not measured stress indicators. Therefore, the present study evaluates the hematology responses of common South American toad *Rhinella arenarum* inhabiting natural and artificial environments associated with a fluorite mine from central Argentina. This species provides a suitable and useful experimental model for monitoring aquatic ecosystems (Vera Candioti et al. 2010, Pollo et al. 2015), and its sensitivity to pollutants was proven in several studies (Howe et al. 1998; Venturino et al. 2003; Bosch et al. 2011; Lajmanovich et al. 2014, Pollo et al. 2015). The study area can be regarded as a "field laboratory" offering an opportunity for the assessment of toxicity under realistic conditions, because of absence of other important sources of contaminants (e.g., agrochemicals, sewage, livestock breeding).

Materials and methods

Study area and site selection criteria

The study area is located in a large granitic batholith, Cerro Áspero (32° 50′ 22.85″ S; 64° 79′40.60″ W; altitude 1200 m.a.s.l) in the center-south region of Sierra de Comechingones, Córdoba, Argentina (Fig. 1). In this area, the main deposits of ephitermal fluorite of Sierras Pampeanas are located (Coniglio 2006). The Sierras Pampeanas are constituted mostly by metamorphic plutonic basement, composed mainly of coarse-grained metamorphic rocks (gneisses and migmatites), and intruded into the Lower Paleozoic by granitic batholiths (Cantú and Degiovanni 1984). These batholiths have an average content of F^- of 1.210 mg/kg, which is two times higher than the host metamorphic rocks and other non-mineralized granites of the Sierras de Córdoba (Coniglio et al. 2006). This natural formation allowed the installation of mines in the area, being the Los Cerros Negros mine the only active in Córdoba since 1991.

The mining establishment is located in a natural matrix in which the remoteness of urban settlements and the characteristics of the mountainous landscape make those potential sources of contamination related to the development of agricultural and livestock activities or sewage absent in the area. The effluents derived from the mineral treatment end in a series of artificial ponds (earth dams) of approximately 15 m by 25 m, elevated about 3 m above the land, vegetated with *Typha* sp. In these artificial ponds, sediments produced by fluorite flotation process are precipitated. These decantation ponds are a closed system, and the effluents never come into contact with nearby natural streams. Associated with this area, the basin of Los Cerros Negros stream, with an area of 10 km^2, circulates on granitic rock from west to east. Near the fluorite mine, it receives the Los Vallecitos stream which is born and runs through metamorphic rock and finally flows into the Guacha Corral River, the most important water course in this area.

The physicochemical characterization of surface water made in this area indicates that fluoride ion is found in concentrations less than 0.35 mg/l for streams belonging to metamorphic environments, while streams circulating in granitic environments have an average concentration of 0.90 mg/l (Coniglio 2006).

Considering the data presented above, three sampling stations were selected: (I) Los Cerros Negros stream (CN), which runs on granitic rock with a high fluorite content; (II) Los Vallecitos stream (LV), which runs on metamorphic rock with low fluorite content; and (III) artificial decantation ponds (DP) containing sediments produced by fluorite flotation process. In all sites, the presence of populations of *R. arenarum* was previously detected (Fig. 1).

Sampling methods and data collection

We sampled all three sites from September 2013 to March 2014, the period of increased reproductive activity for *R. arenarum* and therefore when the individuals are found near of water bodies (Bionda et al. 2011). These months coincide with the period of rainfall and warmer temperatures. In each site, 20 adult individuals were found by visual encounter surveys (Heyer et al. 1994) and captured by hand. To each individual, we recorded snout–vent length (SVL) using a digital caliper Mahr 16 (0.01 mm) and sex according to external secondary sexual characteristics such as the presence of vocal sac and nuptial pads (Duellman and Trueb 1994).

At each sampling site, water samples for chemical analyses were collected. Superficial water (0.25 m depth) samples were collected in 1-L plastic bottles, which were filled to the brim and tightly capped to exclude oxygen preventing oxidations. We also had special care taken to avoid the possible resuspension of sediments. Samples were analyzed for Cl^-, Na^+, K^+, Ca^{2+}, Mg^{2+}, F^-, SO_4^{2-}, and hardness (based on calcium and magnesium total content) by the area of Hydrology, Department of Geology, National University of Río Cuarto, using standard methods (APHA-AWWA 1999). Furthermore, water temperature, pH, electrical conductivity, total dissolved solids (TDS), and salinity were measured in situ, using a digital equipment 35-Series 35425-10 tests (Oakton Instruments

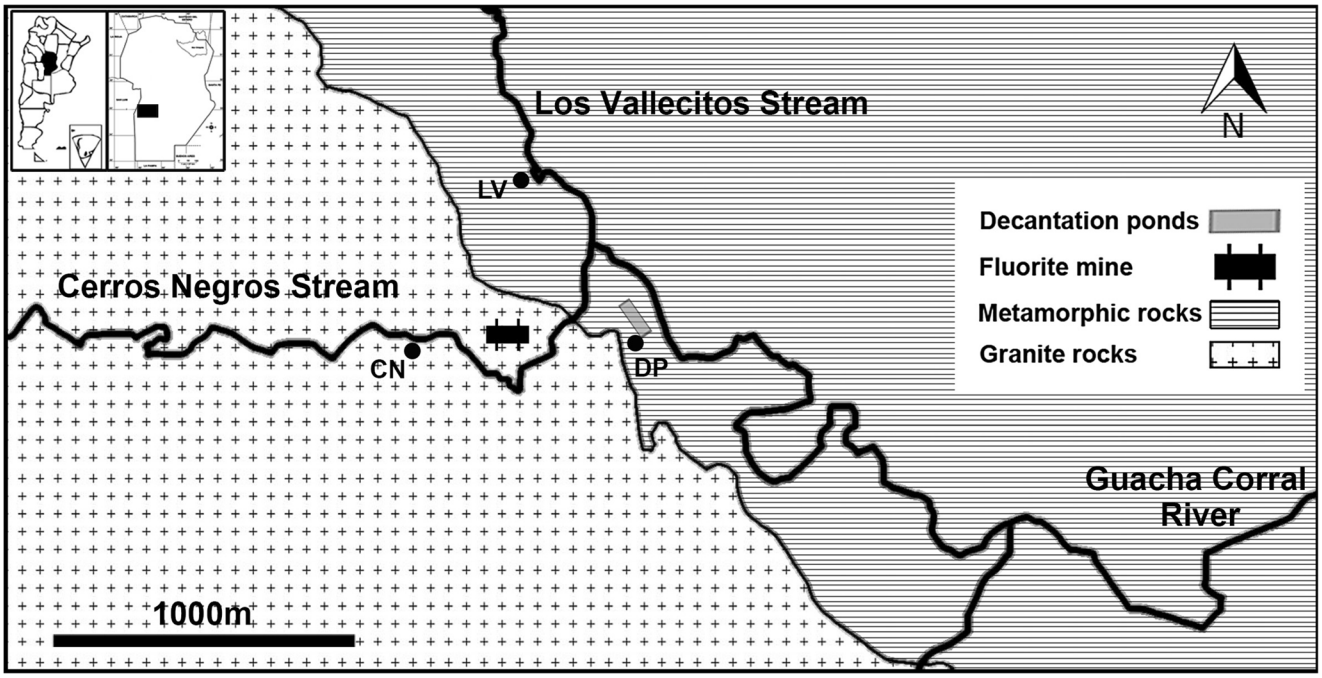

Fig. 1 Location of sampling sites in granitic batholith, Cerro Áspero in the center-south region of Sierra de Comechingones, Córdoba, Argentina. *Black point* indicates the sampling sites

625E Bunker Court Vernon Hills, IL 60061, USA). Dissolved oxygen was measured using a meter HD3030.

Blood cell morphology

Blood samples were obtained from angularis vein of each individual (Nöller 1959; Martino and Sinsch 2002) without sacrificing specimens. Then, all individuals were released at their point of capture. Two peripheral blood smears by each individual were prepared on clean slides, fixed, and stained using May Grunwald-Giemsa (Dacie and Lewis 1984). Two thousand erythrocytes per individual were examined at ×1000 magnification (Zeiss™ Primo Star iLED).

The criteria for distinguishing a MN were as follows: (a) the intensity of a stained MN was similar to that of the principal nucleus but with an inferior diameter, (b) it was round with a nuclear membrane and not connected to the principal nucleus, and (c) did not overlap with the principal nucleus and located within the cytoplasm (Schmid 1975; Fenech 2000). Four ENA were classified according to Carrasco et al. (1990) as blebbed (nuclei presenting a relatively small evagination from the envelope, which seems to contain euchromatin); lobed (nuclei with evaginations larger than those from blebbed nuclei), notched (nuclei that presented a remarkable notch containing nuclear material), and binucleated. The results were expressed as ENA mean frequency (‰) of the sum of all abnormalities observed (Lajmanovich et al. 2014). Furthermore, the mitotic index (MI) was calculated (mitotic cells per 1000 cells).

Immature erythrocyte frequency (IE) was estimated in order to assess alterations on hematological dynamics. The distinction between mature (ME) and immature erythrocytes (IE) was made following Guilherme et al. (2008): IE has a bluish-gray cytoplasm and the nucleus is rounder and larger than ME.

In addition, we performed a differential leukocyte count (neutrophils, eosinophils, basophils, lymphocytes, and monocytes) according to the methodology of Davis (2009). The neutrophils/lymphocyte ratio (*N*/*L*) was calculated as a stress response estimator (Davis et al. 2008).

Statistical analysis

Data distributions for normality (Shapiro-Wilks test) and homogeneity of variances (Levene test) were assessed. A comparative analysis of the environmental variables was performed. Water temperature and pH were compared using one-way ANOVAs followed by the DCG post hoc test (Test Di Rienzo, Guzmán, and Casanoves). Conductivity, salinity, and total dissolved solids were compared between sites using a non-parametric Kruskal-Wallis test, because these variables did not meet the assumptions of the ANOVA.

Significant differences between sites for SVL, MN, ENA, and MI were analyzed using *t* test. Non-parametric Kruskal-Wallis test was used to assess differences between sites in leukocyte. All analyses were conducted using InfoStat (Di Rienzo et al. 2012). Statistical significance was considered to be reached at P value <0.05.

Results

Physicochemical parameters and ion concentrations in water of each sampling site are shown in Table 1. Within each site, there was no significant difference when comparing the SVL between sites (Table 2). However, significant differences in SVL between LV and CN individuals were found ($p < 0.05$), but no differences were recorded between both LV and DP ($p = 0.07$) and between CN and DP ($p = 0.95$) individuals (Table 2) when we do not consider sex.

Sex influence on MN, ENA, and IE frequencies, using SVL as covariable, was evaluated but it was not statistically significant (LV: MN $p = 0.34$, ENA $p = 0.12$, IE $p = 0.35$; CN: MN $p = 0.10$, ENA $p = 0.66$, IE $p = 0.28$; DP: MN, $p = 0.76$, ENA $p = 0.51$, IE $p = 0.92$). Consequently, for subsequent analyses, we combined males and females in a single sample per site.

Analyses of MN frequency and ENAs, considering the total number of nuclear abnormalities (Table 3A), revealed significant differences between sites, except ENAs between CN and DP ($p = 0.15$). LV toads showed the lowest MN and ENA frequencies, while DP was the site with highest frequencies. For immature erythrocytes, no differences were recorded between sites (Table 3A). The dividing erythrocytes, more frequent in toads from DP, appeared mainly as mitotic figures (Fig. 2).

The results of differential leukocyte blood counts are shown in Table 3B. Kruskal-Wallis test showed a significant difference in neutrophils (Fig. 2) and *N*/*L* ratio between sites ($p < 0.05$).

Discussion

Physicochemical characterization of sites and its influence on organisms

Mining activity is a source of physical, chemical, biological, and landscape alterations. Evaluation of environment quality, particularly in aquatic ecosystems, has traditionally been based on physicochemical measurements of water but not necessarily provides adequate information on exposure and response of living organisms to pollution (Antunes et al. 2008; Lavoie et al. 2012), although they are a complementary tool.

Toxicity levels of many pollutants depend primarily on pH, in addition to the temperature (Cairns et al. 1975; Boyd 1982; Hoffman et al. 2010). Aquatic organisms require a pH range between 6.5 and 8 for optimal growth and survival. Outside this range, organisms become physiologically stressed (Addy et al. 2004). In amphibians, pH values for

Table 1 Chemical, physical, and ion concentration data for each sampling site

	Sites		
	LV	CN	DP
Water temperature (T°W)	19.17 ± 2.73	20.01 ± 2.60	23.20 ± 4.15*
pH	8.30 ± 0.24	7.70 ± 0.34	8.50 ± 0.32*
SDT (ppm)	78.57 ± 16.16	35.51 ± 17.43	251.19 ± 374.78*
Salinity (S) ppm	54.75 ± 10.61	26.46 ± 8.92	476.23 ± 382.01*
Conductivity (Cond) µS/cm	112.50 ± 24.50	49.04 ± 23.48	1495.81 ± 643.99*
Dissolved oxygen (O_2) %	92.30 ± 17.18	94.90 ± 12.16	69.93 ± 9.07
Hardness CO_3Ca (ppm)	54.00 ± 5.66	20.00 ± 5.70	74.00 ± 8.50
CO_3 mg/l	1.80 ± 2.55	0.00 ± 0.00	0.00 ± 0.00
HCO_3 mg/l	70.65 ± 7.99	22.50 ± 7.10	375.00 ± 240.40
Sulfates (SO_4^-) mg/l	25.00 ± 0.28	8.25 ± 1.60	102.05 ± 30.20
Chloride (Cl^-) mg/l	3.60 ± 0.99	2.9 ± 0.00	378.6 ± 232.40
Sodium (Na^+) mg/l	7.30 ± 0.99	5.65 ± 1.50	422.65 ± 277.40
Potassium (K^+) mg/l	0.85 ± 0.21	0.30 ± 0.00	9.60 ± 2.10
Calcium (Ca^{++}) mg/l	15.6 ± 5.09	4.40 ± 0.60	18.40 ± 1.10
Magnesium (Mg^{++}) mg/l	3.65 ± 1.77	2.20 ± 1.00	6.85 ± 2.80
Fluoride (F^-) mg/l	0.25 ± 0.07	1.90 ± 1.00	14.20 ± 3.70
Nitrate (NO_3^-) mg/l	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Nitrite (NO_2^-) mg/l	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Mean ± standard deviation

LV Los Vallecitos stream, *CN* Los Cerros Negros stream, *DP* decantation ponds

*$p < 0.05$

Table 2 Snout–vent length of males and females of *Rhinella arenarum* for each sampling site

Sites	*n*	SVL (mm)
LV	6 ♂	96.47 ± 12.72
	14 ♀	90.75 ± 16.75
CN	12 ♂	87.85 ± 8.29
	8 ♀	95.66 ± 7.56
DP	19 ♂	91.42 ± 11.36
	1 ♀	96.20 ± 0.00

Mean ± standard deviation

LV Los Vallecitos stream, *CN* Los Cerros Negros stream, *DP* decantation ponds , ♂ = male; ♀= female

normal development are between 6.3 and 7.7 (García and Fontúrbel 2003). In our study, pH differed among sites with DP showing the most basic values, well above of optimal limits to aquatic organisms.

High concentration of $SO4^{2-}$ found in DP could be a direct outcome of the mining activity (Cañedo Argüelles et al. 2013). During the process, sludge coming out of the plant of flotation is treated with flocculants and sulfates to clarify the water (Martinez J.M., personal communication).

High salinity values recorded in the decantation ponds are mostly the result of the following major ions: Na^+, Ca^{2+}, Mg^{2+}, K^+, Cl^-, $SO_4{}^{2-}$, $CO_3{}^{2-}$, and $HCO_3{}^-$ (Cañedo Argüelles et al. 2013). A high salinity directly influences freshwater organisms because they need to maintain an internal osmotic pressure relative to the medium in which they live. Therefore, when the salt concentration of the medium becomes too high, the osmoregulatory mechanisms could collapse resulting in cellular damage and possibly death (Cañedo Argüelles et al. 2013).

Water quality of sampling sites was evaluated by comparing the results of chemical parameters with globally suggested values for the protection of aquatic biota (Camargo 2003; Canadian Council of Ministers of Environment 2002) and maximum recommended values for human consumption available in the Argentine Food Code of 2012. F^- content at all sites were above the limit suggested (0.12 mg/l) by the Canadian Council of Ministers of Environment (2002). CN stream and DP showed higher concentrations than the value proposed (0.5 mg/l) by Camargo (2003) for protection of aquatic biota in freshwater ecosystems and boundary suggested (1.4 mg/l) by the Argentinian government (Law 24051 1992). Some authors report that fluoride ions act at cellular level as an inhibitor of enzyme activities and causing cell death by apoptosis (Camargo 2003; Barbier et al. 2010), but genotoxic effects are poorly known.

On the other hand, high concentrations of Cl^- and Na^+ in decantation ponds were registered. Exposure prolonged to values above of the proposed limit for protection of aquatic biota could cause developmental abnormalities such as decreased growth and failure or delays in the metamorphosis of amphibians (Sanzo and Hecnar 2006; Collins and Russell 2009; Karraker and Ruthig 2009).

Table 3 Mean values and standard deviations of cytogenotoxicity parameter and leukocyte count of *Rhinella arenarum* individuals

	Sampling sites		
	LV	CN	DP
Cytogenotoxicity parameter			
Micronuclei (MN)	0.03 ± 0.11	0.13 ± 0.29	0.30 ± 0.48 *
Erythrocyte nuclear abnormalities (ENA)	4.79 ± 3.19	8.79 ± 5.80	11.73 ± 7.15**
Mitotic index (MI)	0.19 ± 0.82	0.11 ± 0.29	0.18 ± 0.40
Immature erythrocytes (IE)	3.85 ± 4.33	8.80 ± 15.98	6.25 ± 8.98
Leukocyte count			
Neutrophils	10.44 ± 7.13	8.60 ± 6.52	33.20 ± 26.80**
Eosinophils	4.00 ± 4.66	3.90 ± 3.60	6.60 ± 5.80
Basophils	3.89 ± 2.26	4.50 ± 3.06	8.70 ± 8.26
Lymphocytes			
Immature	17.22 ± 15.16	16.50 ± 15.26	8.20 ± 6.23
Mature	74.00 ± 25.86	69.40 ± 22.71	55.20 ± 25.60
Monocytes	1.44 ± 1.51	2.10 ± 2.60	1.30 ± 1.57
N/L	0.11 ± 0.06	0.11 ± 0.10	0.97 ± 1.38**

Mean ± standard deviation

LV Los Vallecitos stream, *CN* Los Cerros Negros stream, *DP* decantation ponds

*$p < 0.05$, **$p < 0.001$

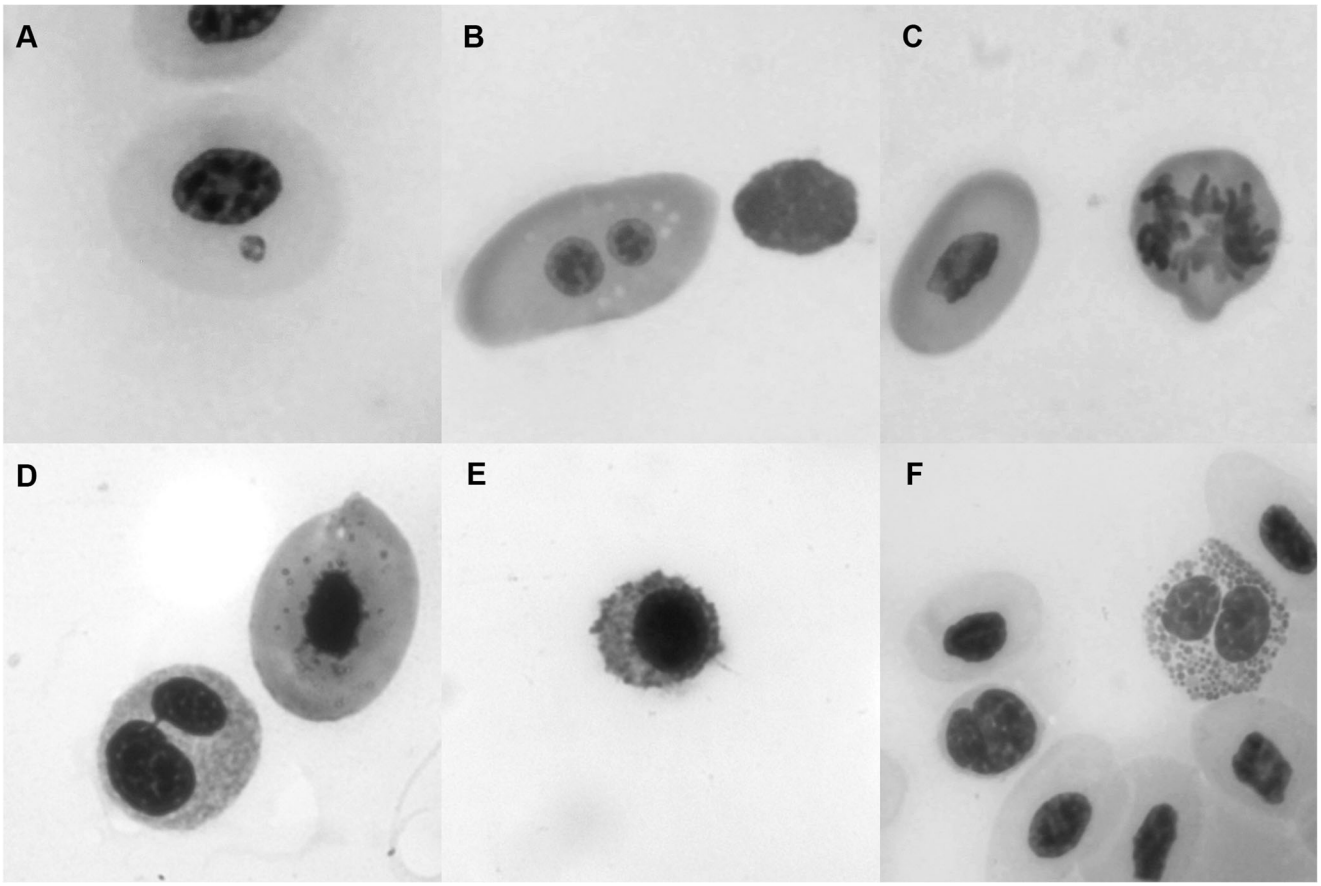

Fig. 2 Erythrocytes and leukocytes in peripheral blood of *R. arenarum.* **a** Micronuclei. **b** Binucleated cell. **c** Mitotic erythrocyte (*right side*). **d** Binucleated neutrophil with cytoplasmatic bridge. **e** Basophils. **f** Monocyte (*left side*) and eosinophils (*right side*)

Cytogenotoxicity and stress

Micronucleus test in erythrocytes is widely used and recommended for studies to cell level on chronic exposure to different types of environmental pollutants with clastogenic and aneugenic properties (Udroiu 2006). According to Serrano García and Montero Montoya (2001), the nuclear abnormalities have a similar origin as micronuclei. For this reason in the last years, they have become very important as potential biomarkers.

In this study, toads from artificial DP and CN stream showed higher frequencies of micronuclei and nuclear abnormalities in comparison to organisms from LV stream. These results suggest genotoxic effects on erythrocytes of *R. arenarum* individuals, most probably caused by chronic exposure to factory effluents (DP) or to natural concentration of fluoride in the stream (CN). These results are in accordance with the studies by Pollo et al. (2016) who observed an increase in the frequency of MN in the ENAs in adults and larvae of *Hypsiboas cordobae*. The presence of micronuclei in a cell reflects structural or numerical chromosomal aberrations arising during mitosis (Fenech et al. 2011). Although there are studies that report variations in the morphology of blood cell in humans (Tolbert et al. 1992; Serrano García and Montero Montoya 2001), mechanisms responsible for ENAs of fishes (da Silva Souza and Fontanetti 2006; Ergene et al. 2007; Pollo et al. 2012) and amphibians (Marques et al. 2009; Lajmanovich et al. 2014; Babini et al. 2015; Pollo et al. 2015) have not been fully understood. Some authors suggested that their high frequencies of apparition may be due to that many xenobiotics may interfere in the DNA synthesis or produce gene mutations in the structural constituents of nuclear envelope of exposed organisms which may result in nuclear anomalies (Beutler 1985; Strunjak Perovic et al. 2009). Other research (Shimizu et al. 1998; Crott et al. 2001) suggested that nuclear budding in interphase (which corresponds to blebbed and lobed nuclei) could be a precursor of micronuclei and represents a process for eliminating amplified genes from the nuclei.

On the other hand, we observed an increase of immature erythrocytes (IE) in toads from DP. It is known that the increase of IE could be a response to stress by pollutant presence (Valenzuela et al. 2006; Prieto et al. 2008). Considering other potential contaminant sources such as agrochemicals, sewage, and livestock breeding are absent in this site, the effluent from the mine could be directly related with this result.

Consequently, the presence of mitotic erythroid cells in polluted conditions indicates that erythropoiesis is directly stimulated in peripheral circulation of adults, which could represent a short-term way for increasing the oxygen carrying capacity of the blood in amphibians (Barni et al. 2007). Additionally, Seriani et al. (2015) found significant increase in the number of erythroblasts, possibly reflecting the release of immature blood cells in response to low concentrations of dissolved oxygen in water.

In addition to effects on erythrocytes, *R. arenarum* individuals from DP showed increased values of eosinophils and *N*/*L* ratio in relation to the other sites. Recent studies performed on amphibians and fishes showed that eosinophil increase may be associated with inflammatory processes and/or antitoxic responses due to either parasite infestation or chemical compounds present in effluents (Romanova and Egorikhina 2006; Barni et al. 2007; Attademo et al. 2013; da Silva Corrêa et al. 2016). In addition, the stress affects the number of neutrophils and lymphocytes in opposite directions and their relationship has been considered as a measure of stress response (Davis et al. 2008; Attademo et al. 2011).

Fluoride toxicity increases with increasing fluoride concentration, exposure time, and water temperature and decreases as water content of calcium and chloride increases and when water hardness is relatively soft (Camargo 2003). In this work, we find that high environmental fluoride correlates with certain responses measured in toads. In decantation ponds, the water had the highest fluoride concentration and mean temperatures and the most basic pH. This could be because it is an artificial environment receiving effluent from an industrial flotation process. During this process, the water is heated to 28 °C, and its pH is modified using calcium carbonate. After the sediment is decanted, the water returns to the factory to restart the process, without making contact with natural water courses. On the other hand, according to Gonzalo and Camargo (2012), the body size of organisms in aquatic medium with high fluoride concentrations has been reported as a factor affecting fluoride bioaccumulation and toxicity, with larger individuals exhibiting a higher tolerance than smaller ones.

Conclusion

Physical–chemical analyses indicated a poor water quality of decantation ponds, as the results revealed ion concentrations which exceed legal standards. Decantation ponds showed higher conductivity, salinity, dissolved solids, and sulfate levels. However, we find toads inhabiting wastewater decantation ponds. Therefore, effluent studies offer a much closer representation of natural exposure scenarios than studies investigating single contaminants (Eggen et al. 2004). In these complex mixtures, the interpretation can be extremely challenging as a result of the multitude of possible synergistic and/or antagonistic interactions between substances, resulting in biological effects that are not easily predictable (Eggen et al. 2004; Gauthier et al. 2004). The mechanisms of chemical synergy for mixtures are not fully understood. Some theories include an increase in the rate of uptake, formation of toxic metabolites, reduction of excretion, alteration of distribution, and inhibition of detoxification systems, which appears to be the most popular theory (Howe et al. 1998). Probably, the choice of this site by the *R. arenarum* individuals is because of the reproductive strategies of this species, which prefers hallow vegetated ponds, with direct sunlight incidence, as oviposition sites, (Sanabria et al. 2007; Bionda et al. 2011). These features could be associated with a higher water temperature and an increase of food and refuge availability.

Finally, this study helps to address an important gap in the literature by expanding on the limited number of in situ studies and exploring impacts of fluoride-mining on amphibians. Nevertheless, more researches are needed to explore the significance of observed effects on amphibian survival and development inhabiting environments impacted by fluoride-mining activities and to continue characterizing responses across a range of sites with different physicochemical components.

Future studies considering other molecular biological tools will be used to distinguish different potential toxicological mechanisms taking into account that fluorine affects different tissues such as the liver, kidney, brain, lung, and testes in animals living in areas of endemic fluorosis (Barbier et al. 2010).

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Compliance with ethical standards

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

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