Ecotoxicology and Environmental Safety 143 (2017) 62–71

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Altered development, oxidative stress and DNA damage in *Leptodactylus chaquensis* (Anura: Leptodactylidae) larvae exposed to poultry litter

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ARTICLE INFO

Keywords:
Poultry production
Biological endpoints
Anuran tadpoles
Organic fertilizer

ABSTRACT

Poultry litter (PL), which is usually used as organic fertilizer, is a source of nutrients, metals, veterinary pharmaceuticals and bacterial pathogens, which, through runoff, may end up in the nearest aquatic ecosystems. In this study, *Leptodactylus chaquensis* at different development stages (eggs, larval stages 28 and 31 here referred to as stages I, II and III respectively) were exposed to PL test sediments as follows: 6.25% (T1), 12.5% (T2); 25% (T3); 50% (T4); 75% (T5); 100% PL (T6) and to dechlorinated water as control. Larval survival, development endpoints (growth rate -GR-, development rate -DR-, abnormalities), antioxidant enzyme activities (Catalase -CAT- and Glutathione-S-Transferase -GST-), and genotoxic effect (DNA damage index by the Comet assay) were analyzed at different times. In stage I, no egg eclosion was observed in treatments T3-T6, and 50% of embryo mortality was recorded after 24 h of exposure to T2. In stages II and III, mortality in treatments T3-T6 reached 100% between 24 and 48 h. In the three development stages evaluated, the DR and GR were higher in controls than in PL treatments (T1, T2), except for those T1-treated larvae of stage II. Larvae of stage I showed five types of morphological abnormalities, being diamond body shape and lateral displacement of the intestine the most prevalent in T1, whereas larvae of stages II and III presented lower prevalence of abnormalities. In stage I, CAT activity was similar to that of control ($p > 0.05$), whereas it was higher in T1- and T2- treated larvae of stages II and III than controls ($p < 0.05$). In stages I and III, GST activity was similar to that of controls ($p > 0.05$), whereas it was inhibited in T1-treated larvae of stage II ($p < 0.05$). T1- and T2-treated larvae of stages II and III caused higher DNA damage respect to controls ($p < 0.05$), varying from medium to severe damage (comet types II, III and IV). These results showed that PL treatments altered development and growth and induced oxidative stress and DNA damage, resulting ecotoxic for *L. chaquensis* larvae.

1. Introduction

The poultry industry is one of the largest growing agro-based industries in the world (Bolan et al., 2010), with fastest development in South America in recent years. Argentina is one of the world's leading producers of chicken meat, with 250,000 million tons of poultry meat exported in 2016 (Haley and Jones, 2016). However, the poultry industry is presently facing different environmental problems, mainly the accumulation of large-scale wastes including manure and litter (Edwards and Daniel, 1992). Poultry litter (PL) is a mixture of feces, wasted feeds, feathers, cereals and rice husks, which is usually recycled and used as organic fertilizer and soil amendment in gardens and agricultural activities (Enticknap et al., 2006; Moore et al., 1995; Wilkinson et al., 2011). Moreover, the manure contains high contents of nutrients (N, P, and K) and organic matter (Moreki and Chiripasi, 2011). Pollution problems occur when PL is applied under environmental conditions in excess of the potential use by the crop or under poor management conditions, causing nutrient loss due to environmental factors such as soil erosion or surface runoff during rainfall (Casey et al., 2006; Kaiser et al., 2009; Sharpley et al., 1998; William et al., 1999). It has been demonstrated that PL accumulates in the soil and that its runoff to nearby water bodies causes eutrophication, with a drastic decrease in dissolved oxygen (Harmel et al., 2004) and several negative effects on aquatic organisms, such as anuran larvae (Mitsch

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http://dx.doi.org/10.1016/j.ecoenv.2017.05.007
Received 27 January 2017; Received in revised form 2 May 2017; Accepted 8 May 2017

and Gosselink, 2000; Peltzer et al., 2013).

In Argentina, the risk of environmental contamination caused by intensive poultry production is potentially increased because the farms are generally concentrated near important river basins, tributaries or streams, and the primary production region (Santa Fe, Entre Ríos, and Corrientes Provinces) is characterized by high rainfall (< 1000 mm^3) and undulating landscapes that can accelerate the runoff and leaching losses. In this context, management practices such as the loading rate and application timing can also play a significant role (Edwards and Daniel, 1992).

In addition, since numerous pharmaceuticals (antibiotics, analgesics and larvicides) are used to control diseases and zoonoses in poultry production (Zhang et al., 2014), they are commonly found in the PL (Carlsson et al., 2009; Larsson et al., 2007; Sims and Wolf, 1994; Teglia et al., 2017). Consequently, through pluvial runoff, these contaminants may end up in the nearest water bodies, affecting soil and water qualities (Wohde et al., 2016).

Some studies have shown that disposal of animal manure in agricultural land could lead to movement of emerging contaminants such as endocrine disruptive drugs into surface and ground water (Peterson et al., 2001; Lee et al., 2007). For instance, dilutions of PL have been demonstrated to delay larval growth and increase female and male gonadal abnormalities in the African frog *Xenopus laevis* (Chen et al., 2013) and to induce feminization in larvae and increase vitellogenin in males of *Pimephales promelas* with a generally dose-dependent response (Yonkos, 2005). Other emerging contaminants such as antibiotics and analgesics commonly used in poultry farming to increase feed efficiency and prevent or treat microbial infections have created public and environmental health concerns (Furtula et al., 2010; Olonitola et al., 2015). Likewise, PL contains metals, such as arsenic, copper and zinc, as results of their use as growth promoters in poultry feed and for prevention of fungal disease (Faridullah et al., 2012; Gupta and Charles, 1999; Jones, 2007; Kpomblecou et al., 2002). Arsenic has affinity for sulfhydryl bonds and can alter protein structure, leading to disruptions of metabolic processes (Gochfeld, 1997).

The runoff of PL and manures also leads to the allogeneic input of viruses and bacteria such as the zoonotic bacteria *Salmonella*, *Campylobacter* and *Escherichia coli* (Jeffrey et al., 1998; Kelley et al., 1994; Ngodigha and Owen, 2009) to the nearest water bodies (Thurston-Enriquez et al., 2005). The presence of antibiotic-resistant bacteria has been widely investigated in both livestock and PL (Furtula et al., 2010; Talebiyan et al., 2014; Zhang et al., 2014). Currently, there are also growing concerns about the presence of antibiotic-resistant pathogens in animal manures from both on-farm exposure and off-farm contamination (Chen and Jiang, 2014).

Amphibians have been extensively used in toxicological tests to assess the toxic effects of contaminants on their growth, development, or reproduction (Feng et al., 2004; Lajmanovich et al., 2003a, 2003b). In this sense, there are no data about the effect of PL on amphibians in Argentina. Studies including the analysis of biomarkers in amphibian larvae exposed to conditions similar to those of aquatic ecosystems provide crucial information for their conservation (Ficken and Byrne, 2012; Peltzer et al., 2013).

This study was designed to characterize for the first time the ecotoxicity of PL on *Leptodactylus chaquensis* (Anura: Leptodactylidae) eggs and two different larval development stages in field experiments. Biological responses were studied based on survival, growth and development, abnormalities, oxidative stress and antioxidant enzyme activities (Catalase -CAT-, Glutathione-S-transferase -GST-) and DNA strand break damages. The information regarding biological exposure/effect assessments and experimental validation using different biomarkers is suitable to characterize the risk of PL on aquatic wild fauna.

2. Materials and methods

2.1. Species selection

Leptodactylus chaquensis (Anura: Leptodactylidae) was used as test organism. This species has an extensive distribution range and abundance in the Neotropical region and is also listed as "not threatened" in the amphibian species categorization of Argentina (Vaira et al., 2012). This species also has a wide environmental plasticity, inhabiting both anthropic and natural environments (IUCN, 2016). Foam nests were collected from a permanent pond (31°43′59″S, 60°31′48″W, Entre Ríos Province, Argentina) by using a net. This pluvial pond was contamination-free, as determined in previous research (Peltzer et al., 2008, 2013), and had 7 mg L^{-1} of dissolved oxygen, pH 7.2, low concentrations of orthophosphate (0.5 mg L^{-1}) and nitrate (0.001 mg L^{-1}), and conductivity of 198 μS cm^{-1}. The foam nests were immediately transferred to the Experimental Module located at Facultad de Bioquímica y Ciencias Biológicas (FBCB) of Universidad Nacional del Litoral (UNL), Santa Fe, Argentina. The experiments were conducted with *L. chaquensis* at three different development stages (Gosner, 1960): eggs (stage 10, eggs with crescent-shape dorsal lip), early larval stage with early limb development (stage 28, larvae with hindlimb bud development) and later larval stage with late limb growth (stage 31, larvae with toe hindlimb differentiation and development). These stages will be referred to as stage I, stage II and stage III, respectively throughout the text, and were selected according to the variation of sensitivity across metamorphosis (Johnson et al., 2011). Such development-variation sensitivity is mentioned in Johnson et al. (2011) to as a 'critical window' in development.

2.2. Experimental design

A chronic and static experiment at mesocosm scale was used to simulate the aquatic systems which receive PL via runoff or lixiviation from the nearest field crop fertilized with PL, from clandestine and unregulated disposal or washed off breeding ground, as observed in preliminary monitoring in the field. PL samples (Fig. 1**A**) were collected

Fig. 1. (A) Poultry litter sample; (B) Poultry litter containers for experiments at mesocosm scale.

in a poultry farm from the locality of Crespo (Entre Ríos Province, Argentina), preserved in sterile, dark polyethylene containers, and then transported in ice to the laboratory in sterile 10-L plastic (high density polyethylene) containers. Sediment tests following the sediment exposure protocols for amphibian larvae were performed to test the ecotoxicity of PL (Peltzer et al., 2013; USEPA, 1996). To replicate the real situation of farm ponds and environmental conditions (temperature, photoperiod, luminosity, and humidity), outdoor mesocosms were used, because they are one of the main standardized methods to assess water or sediment toxicity (Boone et al., 2007; USEPA, 1996, 2007). Mesocosms allow assessing direct and indirect toxic effects on a suit of aquatic organisms present in the test system and are used in risk characterization (Phase I, Toxicity identification evaluation-TIE guidance, USEPA, 2007) in risk assessment (Campbell et al., 1999), because they provide relevant ecological data and mechanistic insight (Evans, 2012).

Mesocosms consisted of glass containers (37 cm long × 20 cm wide × 25 cm deep) covered with iron frames fitted with a 2 × 2-mm mesh to avoid predation or oviposition by insects or other anuran species colonists (Fig. 1**B**). The dilution/proportion PL treatments recommended for TIE (USEPA, 2007) were the following: 6.25% (T1); 12.5% (T2); 25% (T3); 50% (T4); 75% (T5); 100% PL (T6); plus a control with only dechlorinated water. In all PL treatments, sterile sand was used to complete the one kilogram (except in the pure PL, T6), necessary for the solid phase and three equal parts of dechlorinated water. Dechlorinated water had the following physical and chemical characteristics: 176 µS cm^{-1}, 6 mg L^{-1} dissolved oxygen, pH 7.5, and water temperature 24 ± 2 °C. PL treatments were left to stand for 24 h, and the supernatants then removed with a mesh (0.2 mm). Each PL treatment and controls were replicated two times, and each was stocked with an equal number of eggs or larvae (60 eggs from stage I, 60 larvae from stage II, and 60 larvae from stage III). During the experiments, the larvae were fed with boiled lettuce *ad libitum* but in equal ration per treatment. The photoperiod and environmental temperature during the experiments were similarly to those in the real environment (L:D 16:8; 28 ± 2 °C) (Peltzer et al., 2013).

2.2.1. Physical-chemical and microbiological parameters

Water pH, conductivity (µm S cm^{-1}), and concentrations of dissolved oxygen (mg L^{-1}), orthophosphate (PO_4 mg L^{-1}) and ammonium (NH^{4+} mg L^{-1}) were recorded with standard digital instruments and Aquamerk® kits in controls and PL treatments (T1, T2, and T3, Table 1) during all the experiment, in the morning (at 10:00 a.m.) once a week, to know the variation in these variables. A preliminary microbiological screening was performed on a pure PL sample (T6, Table 1) (Cátedra de Microbiología y Biotecnología, Departamento de Ingeniería Alimentaria, UNL). Likewise, emerging contaminants (antibiotics, insecticides and two analgesics) from the PL sample (T6) were considered (Teglia et al., 2017, Table 1).

2.3. Biological responses

Different biological endpoints (survival, growth, development, antioxidant enzyme activities and DNA damage by comet assay) were recorded and monitored at different times, due to the variation in development stages as shown in Fig. 2.

2.3.1. Survival, growth and development

Mortality was recorded at 24 h (day 1) and at the end of each experiment, considering the larvae taken for the analysis of biomarkers (Fig. 2). All treatments were checked daily through the experimental period and the dead individuals were removed and counted. After the beginning of the experiment (day 1), and every five days, five individuals of each treatment were randomly collected, euthanized by immersion in a solution of 0.1% tricaine methanesulfonate (TMS, MS-222) buffered to pH 7.8 with NaHCO3 following the protocol of the Animal Euthanasia Guide proposed by the Institutional Animal Care and Use Committee (IACUC) and the bioethical committee of the FBCB-UNL and fixed in 10% formalin.

Table 1
Physical-chemical variables (treatments, T1-T3), emerging contaminants (chemicals) and microbiology (T6) of poultry litter (PL) obtained from Crespo locality (Entre Ríos, Argentina) used for treatments.

Mesocosms treatments	Poultry litter
Physical- chemical parameters	**Mean ± Standard error (T1, T2, T3)**
Water temperature °C	28 ± 4
Dissolved oxygen mg L^{-1}	2 ± 0.1
pH	8.5 ± 0.1
Conductivity µm S cm^{1}	860 ± 130
Ammonium NH^{4+} mg L^{-1}	400 ± 1.25
Ortophosphate PO_4 mg L^{-1}	10 ± 1.45
Chemicals	**Detected concentration (T6)**[a]
Enrofloxacin µg g^{-1}	0.81
Imidacloprid µg g^{-1}	0.54
Flunixin µg g^{-1}	0.63
Diclofenac µg g^{-1}	0.48
Microbiology	**Detected values (T6)**
Total Coliform Bacteria MPN 100 ml^{-1}	> 110000
Coliform fecal Bacteria MPN 100 ml^{-1}	1.7×10^4
Escherichia coli NPM 100 ml^{-1}	1.7×10^4
Total aerobic mesophilic bacteria CFU 100 ml^{-1}	5×10^7
Enterococcus CFU 100 ml^{-1}	3×10^7
Sulphite reducing bacteria CFU 100 ml^{-1}	> 103
Staphylococcus aureus presumptive CFU ml^{-1}	5.8×10^7

ND no data; MPN Most Probable Number; CFU colony forming units.
[a] Teglia et al. (2017).

The development stages of each preserved specimen were determined according to Gosner table (Gosner, 1960) using a light stereoscope (Leica EZ4 D). Total length (TL) and snout vent length (SVL) were measured using a digital caliper (0.01 mm precision), while an electronic balance (0.01 g precision) was used to estimate body dry weight (BW). The development rate (DR) and growth rate (GR) were calculated according to Teplitsky et al. (2003), as follows: DR: final stage - initial stage/number of days of the experiment; GR: exp (ln (final weight – initial weight)/number of days of the experiment). These specimens were preserved and deposited at the Herpetological Collection of the FBCB-UNL.

2.3.2. Morphological abnormalities

Each preserved specimen was also scored for abnormalities in body shape (diamond body shape), intestinal abnormalities (intestine uncoiling, lateral displacement of the intestine), oral disc (decrease in keratodonts), and axis (stiff tails) (Krishnamurthy and Smith, 2011; Lenkowski et al., 2008). Emaciated body surface and the lack of a smooth-oval contour of the body shape acquired on an angular form were cataloged as "diamond body shape". Stiffness in the tail associated with curved tail compared to control tadpoles was classified as "stiff tail" (Peltzer et al., 2013).

2.3.3. Enzymatic assays

Ten surviving larvae from the different PL treatments (stages I and II from T1 and stage III from T1 and T2) and controls were randomly selected to determine CAT and GST enzyme activities at 168 h (Peltzer et al., 2013). CAT activity was measured using the method described by Aebi (1984), and expressed as µmol H_2O_2 µmol min^{-1} mg^{-1} protein, using a molar extinction coefficient of H_2O_2 40.10^{-3} L mol cm^{-1}. The reaction medium was 50 mM phosphate buffer (pH = 7.2) and 30 M H_2O_2 and absorbance was read on a spectrophotometer at a wavelength of 240 nm at 25 °C (quartz cuvette). GST activity was determined spectrophotometrically using the method described by Habig et al.

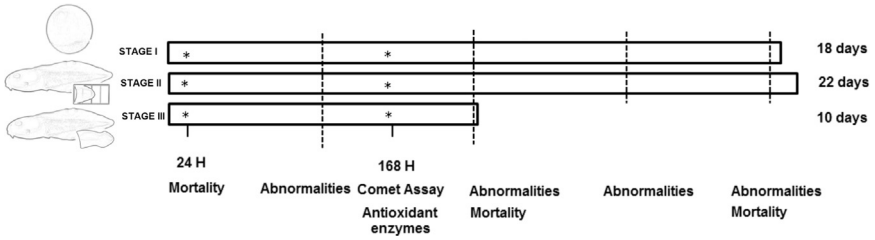

Fig. 2. Temporal overview of the experimental studies according to three development stages (eggs, larval stages 28 and 31, referred to throughout the text as stages I, II and III respectively) and biological responses recorded in *L. chaquensis*. Each discontinuous line reflects five days. The illustration of the development stages was taken from Gosner (1960).

(1974) and adapted by Habdous et al. (2002) for mammal serum GST activity. The enzyme assay was performed at 340 nm in 100 mM Na-phosphate buffer (pH 6.5) [Final volume = 920 µL]), 20 µL of 0.2 mM 1-chloro-2,4-dinitrobenzene, 50 µL of 5 mM reduced glutathione, and the sample. Enzyme kinetics assays were performed at 25 °C and whole GST activity was expressed as µmol min^{-1} mg^{-1} protein, using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}.

2.3.4. Comet assay

At 168 h of exposure, six larvae from each PL treatment (stages I and II from T1 and stage II from T1 and T2) and controls were randomly selected to analyze DNA damage through the Comet assay (in duplicate). The blood of each larva was taken by sectioning behind the operculum and collected with a heparinized capillary tube of 50 µL. The Alkaline Comet Assay (pH > 13) was performed according to the method described by Singh et al. (1988), with the following modifications by Poletta et al. (2008), blood samples were diluted 1:19 (v/v) with PBS medium and used immediately. Then, 2 µL of each diluted blood sample (approximately 4.0×10^3 erythrocytes) was added to 100 µL of 1% low melting point agarose and a slide was prepared. To lyse the cellular and nuclear membranes of the embedded cells, the key-coded slides were immediately immersed in freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM trizma base, 1% Triton X-100 and DMSO 10%; pH 10) and left at 4 °C overnight. The slides were then immersed in freshly prepared alkaline electrophoresis solution (300 mM NaOH and 1 mM Na_2 EDTA; pH > 13), first for unwinding (10 min) and then for electrophoresis (0.7–1 V cm^{-1}, 300 mAmp, 10 min at 4 °C). All the steps were carried out under conditions of minimal illumination and low temperature (on ice). Once electrophoresis was completed, the slides were neutralized and dehydrated with ethanol. Slides were stained with acridine orange at the moment of analysis and 100 randomly selected comets from each animal were visually classified into five comet types according to tail size and intensity (0 = undamaged, I = low damage, II = medium damage, III = moderate damage, and IV = severe damage). Data are expressed as the percentage of damaged erythrocytes and the DNA damage index (DI = n1 + 2.n2 + 3.n3 + 4.n4, where n1, n2, n3 and n4 were the number of cells in each class of damage, respectively) was quantified per treatment (Rodríguez Ferreiro et al., 2002).

2.4. Statistical analysis

For each statistical analysis, data distributions for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene test) were assessed. No differences in larval mortality were found within replicates of each PL treatment (Fisher's exact probability test $p > 0.05$) performed at the different development stages evaluated; therefore, data from replicates of each treatment were pooled for all statistical analyses. The effect of PL treatments at each larval stage analyzed and the variations in each biological response were analyzed individually due to the use of different units and times of data collection. MANOVA (Wilks' lambda multivariate test statistic) was performed to determine whether there were significant overall differences in physical and chemical variables among PL treatments, and subsequent univariate analysis of variance (ANOVA) test followed by Dunnett's post hoc-tests were done. The Chi-square test ($\chi 2$) was performed to assess differences in mortality proportion between PL treatments and controls. The significance level used was $p < 0.05$. The ANOVA followed by Dunnett's post-hoc test was used to compare the SLV, TL and BW of individuals exposed to PL with those of control individuals. Prior to the ANOVA, Levene's tests were used to check the homogeneity of variance, and square transformation of the data was performed when it was necessary to meet the ANOVA requirements of normal distribution and equal variance. Ordinal or nonparametric data were analyzed using the Kruskal-Wallis (KW)-statistic. The prevalence (P) of each morphological abnormality was calculated according to the following equation: number of larvae with X abnormality/total number of larvae analyzed (Peltzer et al., 2013). CAT and GST activities were analyzed at each larval development stage with nonparametric tests, KW-statistic followed by Dunn's post-hoc test or Mann Whitney *U*-test (U). The same procedures were used to analyze the DNA damage index. All the statistical analyses were performed using IBM SPSS Statistics version 21.

3. Results

3.1. Physical-chemical and microbiological parameters

The means of the physical and chemical variables in treatments T1, T2, and T3 are summarized in Table 1. Orthophosphate and ammonia were not recorded in controls. Statistical differences between controls, T1, T2, and T3 (F lambda $\Lambda = 171.173$; $p > 0.05$) were observed. Indeed, PL treatments differed mainly in conductivity, dissolved oxygen, orthophosphate and ammonium (Dunnett's post hoc-test $p < 0.05$). Values of bacteria and chemicals are shown in Table 1.

3.2. Survival, growth and development

In stage I, 100% of egg eclosion was observed within 24 h in control and T1. In T2, only 50% of egg eclosion was observed within 24 h, but they did not survive. In T3 to T6, no egg eclosion was observed. Mortality differed significantly ($\chi 2 = 81.87$; $p < 0.05$) between T1 and T2 at the end of the experiment (18 days). SVL ($F = 0.30$, $p > 0.05$), TL ($F = 0.01$, $p > 0.05$) and BW ($F = 2.32$, $p > 0.05$) showed no significant differences between treatments and controls (Dunnett's post hoc-test $p > 0.05$). T1-treated larvae of stage I showed a lower DR and GR than control larvae. The maximum stage (33) was reached by control larvae (Table 2).

In stage II, mortality reached 100% from T2 to T6 after the first 24 h. Significant differences were recorded for mortality between treatments ($\chi 2 = 46.58$, $p < 0.0001$), reaching a higher value in T1 (50%) at the end of the experiment (22 days). T1-treated larvae of stage II showed higher values of SVL ($N = 46$, $KW = 41.36$, $p < 0.05$), TL ($N = 46$, $KW = 44.73$, $p < 0.05$), and BW ($N = 46$, $F = 20.12$, $p < 0.05$) than controls (Dunnett's post hoc-test $p < 0.05$). The DR and GR in T1-treated larvae of stage II were higher than in control and T2-treated larvae (Table 2).

In stage III, mortality was higher than 90% from T3 to T6 at 24 h.

Table 2
Survival, growth and development, morphological abnormalities, antioxidant enzymes activities, and DNA damage of *L. chaquensis* exposed to poultry litter (PL) treatments and controls at different development stages (eggs, larval stages 28 and 31, referred to throughout the text as stages I, II and III respectively). T3-T6 PL treatments were discarded because mortality reached 100% within 24 h.

	Poultry litter experiments by development stage								
	Stage I			**Stage II**			**Stage III**		
	Control	**T1**	**T2**	**Control**	**T1**	**T2**	**Control**	**T1**	**T2**
Mortality (Percentages)									
24 h	0	0	50	0	0	95	0	0	0
End of experiments	15	100	100	25	50	100	15	100	95
Growth and development parameters									
SVL (mean ± SD)	4.89 ± 0.83	5.14 ± 0.61	ND	7.14 ± 1.79	9.18 ± 2.01	5.59 ± 0.79	16.44 ± 10.12	12.63 ± 1.18	12.99 ± 2.41
TL (mean ± SD)	12.11 ± 2.26	11.69 ± 1.83	ND	18.67 ± 1.79	25.45 ± 1.75	10.81 ± 1.84	30.91 ± 1.15	31.14 ± 3.41	34.35 ± 6.14
BW (mean ± SD)	0.03 ± 0.02	0.02 ± 0.01	ND	0.07 ± 0.06	0.18 ± 0.09	0.02 ± 0.01	0.29 ± 0.18	0.24 ± 0.06	0.33 ± 0.18
Growth rate	0.95	0.91	ND	0.92	0.93	0.84	0.82	0.60	0.62
Development rate	1.27	1.05	0.83	0.45	0.63	0.09	1.4	0.3	0.6
Maximum stage reached	33	29	25	38	42	30	45	34	37
Abnormalities (Prevalence)									
Diamond body shaped	ND	0.15	ND	ND	0.06	ND	ND	0.12	0.06
Intestine uncoling	ND	0.08	ND	ND	ND	ND	ND	ND	ND
Lateral displacement Intestine	ND	0.18	ND	ND	0.15	ND	ND	ND	0.03
Stiff tail	ND	0.08	ND	ND	ND	0.02	ND	0.03	ND
Oral disc alteration	ND	0.04	ND	ND	ND	0.08	ND	ND	ND
Antioxidant enzymes									
CAT μmol min^{-1} mg^{-1} TP	11.27 ± 6.77	21.6 ± 9.11	ND	14.35 ± 1.54	32.95 ± 8.03	ND	14.54 ± 1.95	31.46 ± 9.80	28.80 ± 2.18
GST μmol min^{-1} mg^{-1} TP	0.73 ± 0.5	0.42 ± 0.11	ND	1.05 ± 0.2	0.69 ± 0.11	ND	0.64 ± 0.21	0.88 ± 0.47	0.73 ± 0.29
DNA damage	ND	ND	ND	Low (comet type I)	Medium and moderate (comet types II and III)	ND	Low (comet type I)	Medium and moderate (comet types II and III)	Moderate and severe (comet types III and IV)

ND no data, CAT catalase, GST gluthatione S-transferase, TP total protein.

No significant differences were observed between treatments ($\chi 2 = 0.9$, $p > 0.05$) in mortality at the end of the experiment (10 days). No significant differences were observed in SVL (N = 33, KW = 1.33, $p > 0.05$), TL (N = 33, KW = 3.04, $p > 0.05$), or BW (N = 33, KW = 1.81, $p > 0.05$) between T1- and T2-treated larvae of stage III and controls (Dunnett's post hoc-test *pT1-T2 vs Control* > 0.05). The DR and GR in controls were higher than in PL treatments (Table 2).

3.3. Morphological abnormalities

T1-treated larvae of stage I presented five types of morphological abnormalities, with diamond body shape (P = 0.15) and lateral displacement of the intestine (0.18) being the most prevalent ones, whereas controls presented no abnormalities (Table 2). Moreover, the presence of abnormalities in treated larval of stages II (T1) and III (T1 and T2) was infrequent, being diamond body shape and lateral displacement of the intestine the most prevalent ones (Table 2; Fig. 3**A–C**).

3.4. Enzymatic assays

No significant differences in CAT (Mann Whitney U-statistic = 17.5, $p > 0.05$) or GST activities (U = 39, $p > 0.05$) were observed between controls and T1-treated larvae of stage I at 168 h. In T1-treated larvae of stage II, CAT activity was significantly higher at 168 h of exposure than in controls (U = 0.00001, $p < 0.0001$) (Table 2, Fig. 4**A**), whereas GST activity was significantly lower than in controls (U = 6, $p < 0.001$) (Table 2, Fig. 4**B**). In T1- and T2-treated larvae of stage III, CAT activity was higher (KW-statistic = 19.53, $p > 0.0001$) than in controls (Dunn's post hoc-test *pT1-T2 vs control* < 0.0001, respectively) (Table 2, Fig. 4**C**), whereas GST activity showed no significant differences (KW-statistic = 2.355, $p > 0.05$) compared to controls (Dunn's post hoc-test $p > 0.05$, Table 2).

3.5. Comet assay

Controls and T1-treated larvae of stage I, no DNA damage was observed in (Table 2). However, in T1-treated larvae of stage II, DNA damage differed significantly from that in controls (U = 0.0008; $p < 0.05$), being comet types II and III (medium and moderate DNA damage) the most frequent (42.1%) (Table 2, Fig. 5**A**). Thus, in T1- and T2-treated larvae of stage III, DNA damage increased significantly (KW = 11.90, $p < 0.001$) respect to controls (Dunn's post-test *pT1 vs control* < 0.05; *pT2 vs control* < 0.01; Fig. 5**B**). Comet type II (medium DNA damage) was the most frequent (44%) in T1, while comet types III and IV (moderate and severe DNA damage) were the most frequent (56.6%, 19.4%) in T2. In controls of larval stages II and III, the most frequent was comet type I (89%) (Fig. 5**C,** Table 2).

4. Discussion

Our study showed lethal and sublethal effects of PL on eggs and larvae of *Leptodactylus chaquensis* under mesocosm conditions. The lethal effect of PL was clearly visible in PL experiments with early development stages (stage I), because no egg eclosion was observed at concentrations greater than that T2. In accordance to this result, a new

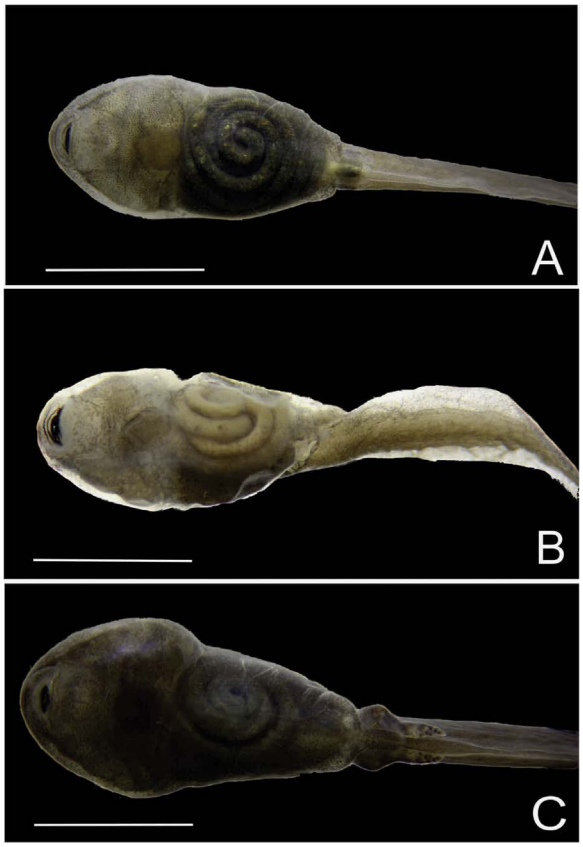

Fig. 3. Examples of some abnormalities of *L. chaquensis* larvae observed in the different poultry litter (PL) treatments. (A) Control larvae; (B) larvae with lateral displacement intestine; (C) larvae with diamond body shape (emaciated). Scale: 3 mm.

dilution series or another dilution factor between T1 and T2 PL treatments should be necessary to determine the LC50. One explanation for this may be the low dissolved oxygen concentrations (1 mg L^{-1}), and excess of ammonium and orthophosphate recorded in the PL treatments respect to controls, as reinforced by the MANOVA and subsequent ANOVA tests. Another possible explanation is the complex environment context, in which decreases in oxygen and increases in nitrogen and phosphorus act synergistically to cause deleterious effects on amphibian larvae (McDaniel et al., 2004; Metts et al., 2012).

Moreover, stress conditions due to physical-chemical variables and emerging contaminants (enrofloxacin, imidacloprid, diclofenac, flunixin), in complex mixture affect early stages of *L. chaquensis* larvae, as demonstrated by other authors (Peltzer et al., 2013, 2017; Shinn et al., 2008). In addition, in this study, we showed that PL represents a reservoir of several bacteria including antibiotic-resistant bacteria (Furtula et al., 2010) such as *Escherichia coli* and *Staphylococcus aureus*, which may produce mortality and diseases (Densmore and Green, 2007) and whose values in PL treatments were risky for aquatic biota (USDA, 2009; USEPA, 2004, 2010). It is important to note that amphibian larvae are suitable hosts of pathogenic bacteria (Grey et al., 2007; Lajmanovich et al., 2001) and only when pathogen numbers increase beyond a threshold, as observed in PL, for which the host can compensate due to intrinsic or extrinsic factors (e.g., environmental, nutritional, immune function), amphibian larvae become infected or diseased (Miller et al., 2009). In this way, the causes of the high mortality of *L. chaquensis* larvae observed in all PL treatments during the experiments are complex, but likely include the interaction of the mentioned stressors, which should be the topic of future investigations.

In the PL treatments, N is present as organic N, ammonium or nitrate forms (Sharpley and Smith, 1995; Sims and Wolf, 1994), exceeding the reference values to protect aquatic biota (Canadian water quality guidelines CEQGs, 2008) and the recommended values to protect water quality (Canadian water quality guidelines CEQGs, 2008; USEPA, 2001, 2013). Numerous evidences indicate the impact of nitrogenous compounds on biological aspects of amphibians (Egea-Serrano and Tejedo, 2014; Krishnamurthy et al., 2006; Shinn et al., 2008). It is known that the excess of nitrates causes a delay in the development of *Rana cascadae* (Marco and Blaustein, 1999), as observed in this study in T1-treated larvae of stage I, as well as morphological abnormalities and unusual swimming patterns in *Bufo bufo* tadpoles (Marco and Ortiz-Santaliestra, 2009; Xu and Oldham, 1997). Furthermore, the excess of N and P in PL can cause the eutrophication of natural water, inducing an increase in the growth of algae or aquatic plants (Mitsch and Gosselink, 2000) and a decrease in dissolved oxygen concentrations (Edwards and Daniel, 1992; Sims and Wolf, 1994), which severely affect the survival, growth and development of amphibian larvae (Peltzer et al., 2008). In larval stages I and III, the mean SVL, TL and BW did not differ significantly between treatments, whereas in larval stage II, *L. chaquensis* larvae were larger and heavier than controls. It may be possible that the high levels of organic particulate matter in PL can serve as food for the species. It has been demonstrated that fish in ponds fertilized with poultry manure grow significantly better than those in ponds fertilized with cattle manure, pig manure or no manure treatments (Kang'ombe et al., 2006).

Morphological abnormalities of amphibian larvae have been linked to the presence of metal trace elements (Haywood et al., 2004; Plowman et al., 1994), nutrients (Peltzer et al., 2008; Wood and Richardson, 2009), agrochemicals (Lajmanovich et al., 2003a, 2003b; Lenkowski et al., 2008, 2010; Peltzer et al., 2011), and emerging contaminants such as pharmaceuticals used in veterinary medicine (Peltzer et al., 2017; Zhang et al., 2014). Except for metals, these stressors were present in our PL treatments, and may explain the presence of morphological abnormalities observed in all PL treatments. Moreover, the prevalence of larval morphological abnormalities increased at the early stages of development (stage I). In this way, *L. chaquensis* individuals exposed to PL treatments early in the growth process have a much greater risk of malformations and mortality, as demonstrated by Johnson et al. (2011). Further studies should be carried out considering the complex interaction among stressors and the critical window in development often referred to as developmental sensitivity (Johnson et al., 2011).

GST and CAT enzyme activities are earlier enzyme biomarkers of oxidative stress caused by different chemicals, including pesticides and pharmaceuticals in native anuran larvae (Attademo et al., 2007, 2015; Peltzer et al., 2017), but no data exist regarding PL contamination. The CAT activity of *L. chaquensis* larvae at stages II and III exposed to PL treatments for 168 h was significantly higher than that of controls. Similarly, we have previously observed higher CAT activity in larvae exposed to sediments from a pond close to an industrial production area and soybean fields in contrast to native forest (control) (Peltzer et al., 2013). In this way, the results clearly suggest PL-induced oxidative stress. The increase in CAT activity in T1- T2 treated larvae of stage II and III is probably a response to PL-induced toxic stress and serves to neutralize the impact of the increased generation of reactive oxygen species (John et al., 2001). Likewise, Li et al. (2010) reported similar findings in the rainbow trout (*Oncorhynchus mykiss*) after chronic exposure to the fungicide propiconazole. The significant increase in CAT activity in *L. chaquensis* larvae at these stages exposed to PL could be attributed to the high pollutant impact (Lushchak, 2011).

The GST activity in T1-treated larval of stage II was inhibited at 168 h respect to controls; whereas that of T1- and T2-treated of stage III and controls was similar. GST reduction is regularly related to the enhancement of peroxidation processes in the cell membrane and leads to stress and can highly contribute to hepatotoxicity (Viarengo et al., 2007). This reinforces the idea of the presence of oxidants that could lead to the inactivation of the enzymatic activity (Bagnyukova et al.,

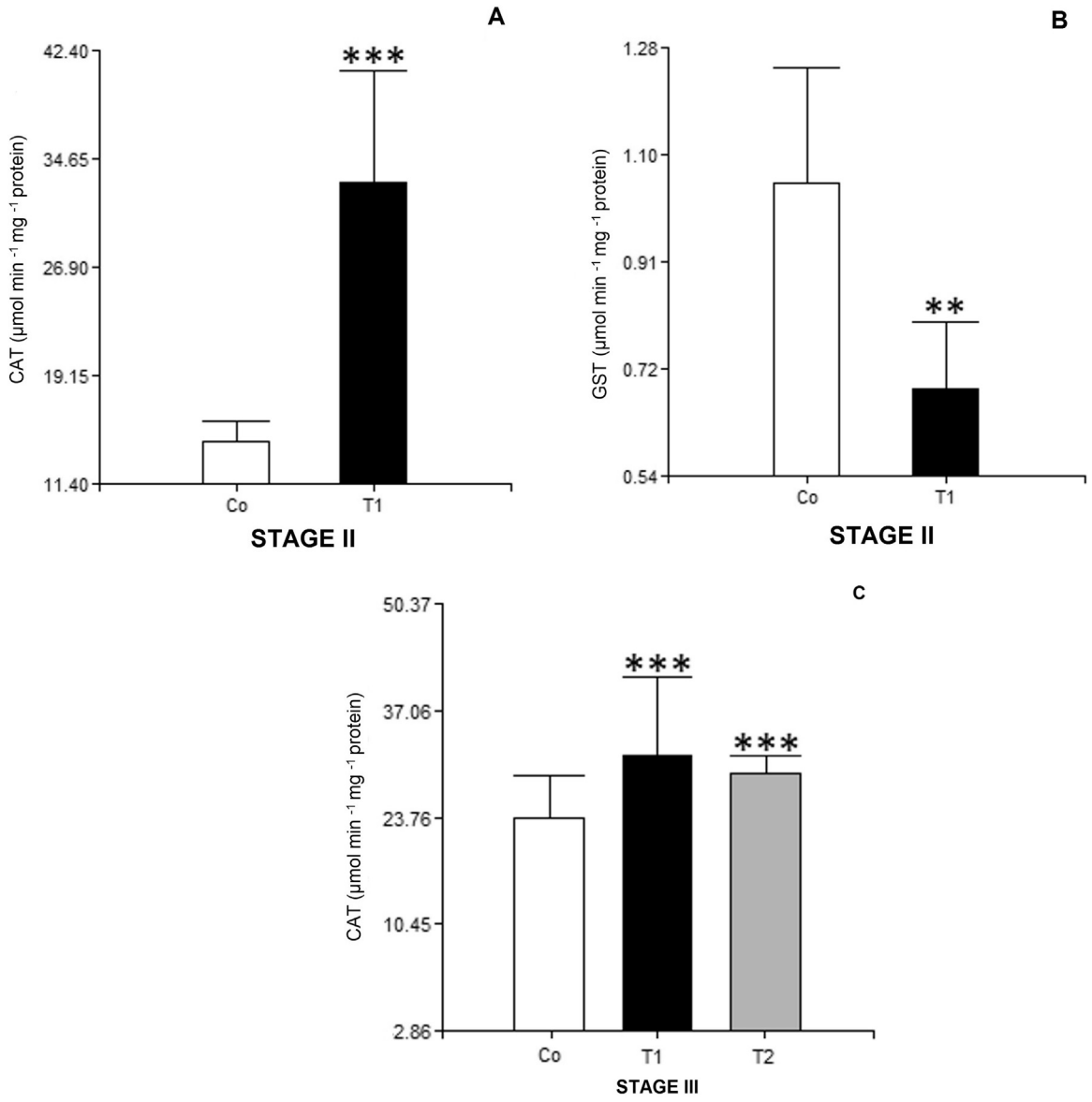

Fig. 4. Catalase (CAT) and Glutathione S-transferase (GST) activities at 168 h in *L. chaquensis* larvae exposed to the following poultry litter (PL) treatments (T1, T2) and Controls. (A) CAT and (B) GST activity in T1-treated larvae of stage II and (C) CAT activity in T1- T2-treated larvae of stage III. Asterisks express significantly different from controls (** $p < 0.001$, *** $p < 0.0001$).

2006; Lushchak et al., 2009) and may be a sign of interference of this antioxidant defense (Modesto and Martinez, 2010). In addition, it is possible that a deficiency in GST activity due to its inhibition was compensated by enhanced CAT activity, as it occurred in T1-treated larval of stage II. The alteration in antioxidant enzymes may be due to the reaction and toxic effect of PL, which may cause oxidative stress in *L. chaquensis* larvae. However, the antioxidant defense may vary from tissue to tissue, presence or formation of liver and kidney (where antioxidant detoxifying processes occur), the protein expressions of CAT or GST through metamorphic development switches or environmental factors (Amicarelli et al., 2004; Gomez-Mestre et al., 2013). The last may explain the differences of such enzymes among development stages exposed to PL (Hansen et al., 2006). Therefore, the assessment of CAT and GST activities should be used in PL monitoring programs for amphibians, being effective methods to determine oxidative stress.

DNA damage has been proposed as a useful parameter to assess the genotoxic properties of environmental pollutants. Medium, moderate and severe DNA damage in T1-treated larvae stage II and T1- and T2-treated larvae of stage III indicated genotoxic effect of PL. It is well known that some DNA breaks are present in vertebrate cells due to normal metabolism and apoptosis, which might explain the DNA damage found in controls of both larval stages (II and III), but further analyses are necessary to determine whether the DNA breaks resulted either from DNA damage by the free radicals produced by cellular oxygen metabolism due to PL exposition or from DNA genetic processes at different developmental stages. In addition, hypersensitivity to DNA damage in the stage II and III is probably due to a cell fate-dependent mechanism to ensure genomic integrity during a period of extreme proliferation, organ remodeling and differentiation (Ishizuya-Oka, 2011). Although chemical-induced DNA damage in amphibians is well documented worldwide (Mouchet et al., 2006), no data are available for the genotoxic effect of PL on native Argentinean amphibians. Increased genomic instability plays an important role in decreased fitness of exposed amphibians and could lead to adverse effects on long-term population survival (Barni et al., 2007; Valencia et al., 2011). The results described here showed that the Comet assay can provide a good estimation of DNA damage in *L. chaquensis* larvae exposed to PL contamination and that it can be used as a suitable DNA damage biomarker in genetic toxicology.

Poultry litter contamination threatens amphibian larvae, and the

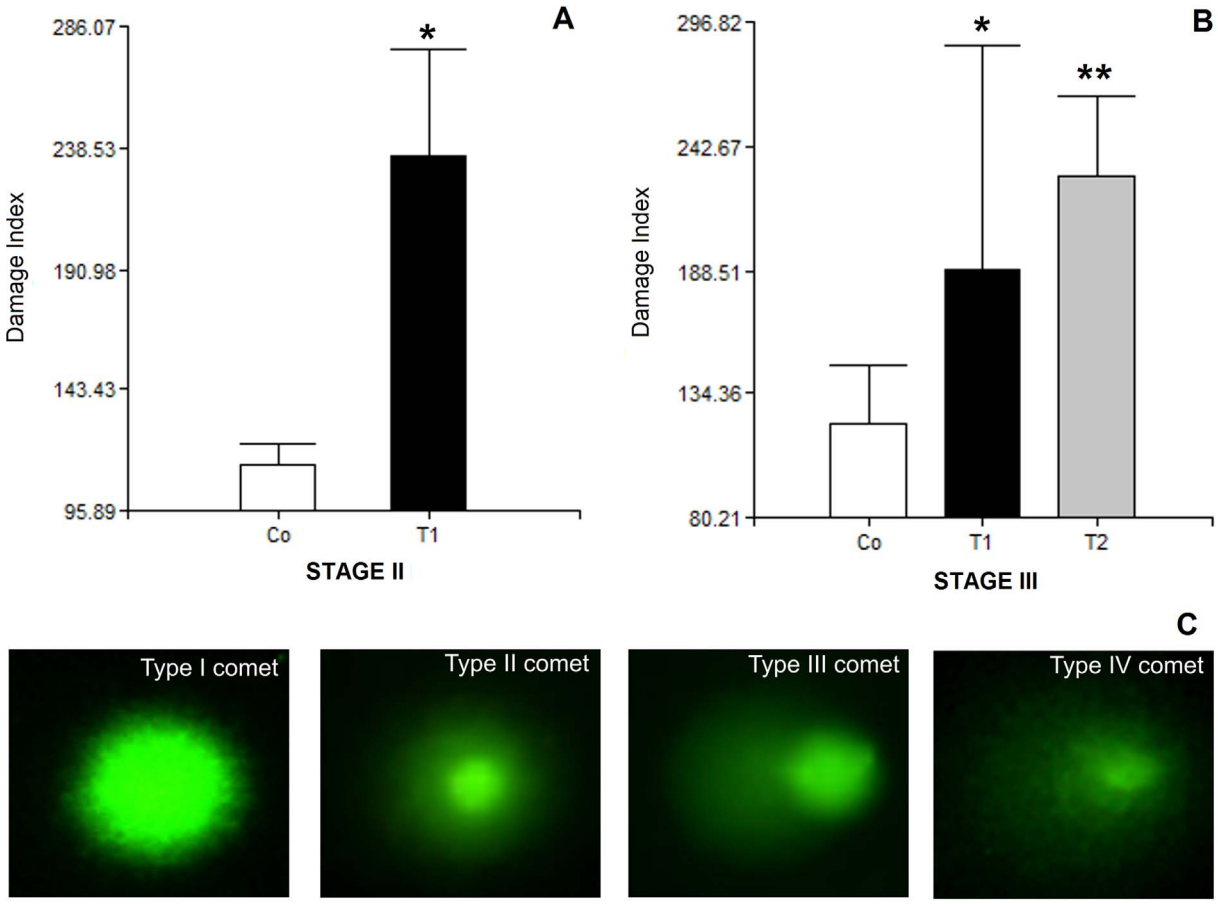

Fig. 5. DNA damage index (based on Comet assays) in *L. chaquensis* erythrocytes at 168 h exposed to poultry litter (PL) (A) T1-treated larvae of stage II and (B) T1- T2-treated larvae of stage III showing Comet types (C). Data are expressed as mean ± SD. Asterisks indicate significantly different from controls (* $p < 0.05$).

negative effects could be due to several causes (complex mixture of physical and chemical variables, and pathogens), with different consequences on biological endpoints (growth and development, antioxidant enzymes and DNA) that could vary with development stages. This novel threat for amphibian wildlife and its effects must be considered in faunal monitoring on intensive poultry production areas, which allow characterizing ecotoxicological risk with several other biomarkers through acute and chronic exposures with single or multiple amphibian species and multiple developmental stages (Phase II and III, TIEs guidance, USEPA, 2007). Finally, it is important to mention that environmental management of poultry residues (such as washing off of poultry feedlots) and their use as organic fertilizers should be regulated, as a consequence of the biological impairments observed in the native amphibian here studied. Considering the results of the present study, it is important to mention that poultry industries in Argentina have increased largely in the last decade and that the release of large amounts of waste and the use of PL as a fertilizer are generating animal, ecological and human health concerns.

Aknowledgements

We thank to Teglia, C., Culzoni,J., Goicoechea H., Russel-White, K., Scarinci, H. E., Carrasco, M.S., Simonetta, A. for provide informations regarding emerging contaminants and bacteria, and performed preliminary analysis. To Grenon P., Junges C. and Basso A. for laboratory assistance. To Eusevi, M.V. for English Editing Service. This work was supported by the Universidad Nacional del Litoral (Project CAI + D N° 045, 2012), CONICET (PIP N° 036, 2014) (Consejo Nacional de Investigaciones Científicas y Técnicas) and ANPCyT-FONCyT (PICT N° 0470, 2014) (Agencia Nacional de Promoción Científica y Tecnológica, Project).

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