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# Evaluation of the toxicity of the sediments from an agroecosystem to two native species, *Hyalella curvispina (CRUSTACEA: AMPHIPODA)* and *Boana pulchella* (AMPHIBIA: ANURA), as potential environmental indicators

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ABSTRACT

In a study area chosen outside the city of La Plata (Buenos Aires, Argentina), two zones were selected having different types and magnitudes of agrarian activity: one represented mainly by grasslands with a low degree of anthropic influence, the other by intensive cultivation under cover with a high level of anthropic impact. The first objective was to assess the toxicity of the sediments from the study areas to two local species and compare the responses of both bioassays (assessing mortality and growth inhibition) in a standardized test protocol. *Hyalella curvispina* (*Hc*) is a validated indicator of sediment toxicity; *Boana pulchella* (*Bp*) has likewise proven useful as a study model through its sensitivity to different pollutants. The comparison was performed by multivariate analyses at the time of evaluating sediment toxicity in a stretch of the Carnaval Stream that flows through an agroecosystem. The second objective was to evaluate the presence of morphologic abnormalities, among other supplemental endpoints (behavior and development), in tadpoles of *Bp* since those features are good indicators of sublethal effects. *Hc* turned out to be the more sensitive to the chemical profile of the agroecosystem. We detected two herbicides, trifluralin and acetochlor, and three insecticides, chlorpyrifos, endosulfan, and lambda-cyhalothrin and in this test proposed *Bp* as a second indicator of sediment toxicity. This research was the first to use larvae of *Bp* to evaluate the toxicity of sediments of a particular site. The analysis of the occurrence of morphologic abnormalities enabled the detection of toxicities in locations where other endpoints remained unaffected. We intend to incorporate this species into sediment testing since *Bp* is a prominent anuran in pampean agroecosystems whose utility in toxicity bioassays has been verified by numerous ecotoxicological studies. In conclusion, both the species *Hc* and *Bp* should be considered appropriate as tests organisms for the assessment of sediment quality as well as effective and promising environmental indicators.

## 1. Introduction

Agriculture is one of the human activities that most affects ecosystems (Paruelo et al., 2005, Lotz, 2009) because of the use of agrochemicals on large tracts of land along with the homogenization of the environment (Devine and Furlong, 2007, Grau and Aide, 2008, Lasier et al., 2016, Suarez et al., 2016). The city of La Plata (Buenos Aires, Argentina) has a periurban green belt (PGB) in which floricultural and horticultural activities are carried out. According to the last census (MAA, 2006), the PGB has an area of 2880 hectares, of which 1888 ha (66%) are open field and 992 ha (34%) are with cultivation under cover (*i. e.*, involving greenhouses), with this zone constituting the largest area in the country occupied by greenhouses (Ringuelet, 2008). Intensive agricultural activity under cover requires a large variety and quantity of pesticides (Pórfido et al., 2014). In the cultivated areas, the permanent use of water along with the generation of ponds, drainage ditches, or irrigation channels provides potential habitats for aquatic and terrestrial taxa (Knutson et al., 2004, Herzon and Helenius, 2008). Ecotoxicological studies conducted in the PGB have detected the presence of several pesticides—including glyphosate and atrazine

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herbicides along with the insecticides chlorpyrifos, cypermethrin, and endosulfan—in samples of soil, water, and sediment from streams that cross the PGB (Camilión et al., 2003, Ronco et al., 2007, Agostini et al., 2013, Mac Loughlin et al., 2017). Specifically, the presence of Acetochlor, Atrazine, Trifluralin, Pendimethalin, Bifenthrin, Cypermethrin, Deltamethrin, Permethrin, Lambda-cyhalothrin, Chlorpyrifos, Diazinon, Malathion, Parathion, Methyl parathion, Fipronil, Endosulfans, α-Lindane, β-Lindane, γ-Lindane, Heptachlor, Heptachlor epoxide (isomer A), Heptachlor epoxide (isomer B), o,p′-DDE, p,p′-DDE, Dieldrin, p′p-DDD, Endrin, o,p′-DDT, p,p′-DDT, Aldrin, Pyraclostrobin, Azoxystrobin, Epoxiconazole, and Tebuconazole was previously determined in the PGB by Mac Loughlin et al. (2017).

When pesticides are used intensively and continuously for a long time, that practice increases the likelihood of those compounds coming into contact and interacting with nontarget organisms (Manahan, 2011). This exposure can drive the components of the ecosystem into a state of imbalance constituting a form of stress (Newman, 2012) that, in turn, generates a response, effect, or detectable and measurable feature at all of the different ecotoxicological levels (Newman, 2014). A suitable tool for determining the effects of physical and chemical agents on test organisms under controlled experimental conditions are tests involving biologic toxicity (*i. e.*, bioassays; Adams, 2003, Castillo Morales et al., 2004). Among the different types of bioassays sediments are considered a useful tool for diagnosing ecosystems (Buikema et al., 1982).

Benthic organisms, in their association with the bottom of water bodies, constitute an essential link in the food webs and are the intermediates between the primary producers and the detritus and top consumers, so that adverse biologic effects produced in these organisms can undermine the quality of the benthic sediments (Wenning and Ingersoll, 2002). Four physical phases of the sediment, each of which reflects a different type of exposure to toxic conditions, can be used to assess pollution: (i) sediment’s interstitial (pore) water (Ingersoll et al., 1995), (ii) the elutriate (*i.e.*, water-extractable) fraction of the sediment (Burton et al., 1995), (iii) the whole sediment, and (iv) the organic extracts from the sediment (True and Heyward, 1990). Of the four phases, the one that best reflects the natural conditions to which the biota present are exposed in the environment is the whole sediment, since that phase enables an evaluation of the bioavailability of contaminants (Landrum and Robbins, 1990, Riba et al., 2004). In general, these bioassays use different organisms, ranging from bacteria and algae to aquatic vertebrates; but the most commonly used test organisms are the benthic invertebrates, with the most frequently used species being the crustacean *Hyalella azteca* (Ingersoll et al., 1995, Kubitz et al., 1996, Sun et al., 2015, Brock et al., 2016). With respect to vertebrates, the larvae of amphibians have also been incorporated as test organisms in standardized protocols (USEPA, 1996, ASTM, 2007). Since the tadpoles of many species are in permanent contact with the sediment, the dermis and the digestive tract can constitute relevant pathways for exposure to contaminants (Wenning and Ingersoll, 2002).

In the evaluation of local ecosystems, a common tendency is to incorporate native and representative species as test organisms in toxicity assays (Semlitsch and Bridges, 2005, Peluso et al., 2011). In recent years, the amphipod *Hyalella curvispina* (*Hc*), widely distributed in South America, has been used as a test organism in ecotoxicological assessments. This amphipod is part of the native fauna and has been used in laboratory bioassays to assess sensitivity to different toxic agents (Giusto et al., 2012, Peluso et al., 2013a, Anguiano et al., 2014, Mugni et al., 2015), in assessments of environmental samples (Di Marzio et al., 1999, Peluso et al., 2011, Míguez et al., 2012, Peluso et al., 2013a, Peluso et al., 2013b, Giusto et al., 2014, Peluso et al., 2016, Mac Loughlin et al., 2017), and as a test organism in certain field evaluations (Graça et al., 2002, Jergentz et al., 2004, Venturino et al., 2007, Mugni et al., 2012, Mugni et al., 2016). Although other species of amphibians have been used in bioassays as potential model indicators (Natale et al., 2006, Pérez-Iglesias et al., 2014, Salgado Costa, 2016); only one protocol in Argentina, under the acronym of ANFITOX, has been found to be applicable to embryos and larvae of amphibians (Herkovits and Pérez-Coll, 1999). One native species, however, that has been employed in several ecotoxicological studies demonstrating its usefulness as a model indicator with respect to sensitivity to different families of pollutants is *Boana pulchella* (*Bp*; Natale et al., 2006, Agostini et al., 2009, Agostini et al., 2010, Junges et al., 2010, Brodeur et al., 2011, Brodeur et al., 2012, Agostini et al., 2013, Peltzer et al., 2013, Pérez-Iglesias et al., 2014, Ruiz de Arcaute et al., 2014, Pérez-Iglesias et al., 2015, Schuch et al., 2015, Pérez-Iglesias et al., 2017, Natale et al., 2018).

Within this context, the first objective in the present work was to assess the toxicity of the sediments from PGB in two local species in order to evaluate and compare the responses of both bioassays, using a standardized test with sediment and a validated species (*Hc*) as indicator of sediment toxicity. Here, we intend to propose the species *Bp*, a comparable model to the rest of the vertebrates, as another validated indicator of sediment toxicity. The comparison was performed by multivariate analyses at the time of evaluating the toxicity of sediments, with the latter being obtained from the Carnaval Stream in a stretch that flowed through an agroecosystem located in the PGB. The second objective was to evaluate the presence of morphologic abnormalities, among other supplemental endpoints (*e.g.*, measurements of behavior and development), in anuran tadpoles of a native species, since anuran larvae constitute good indicators of sublethal effects. The relevance of evaluating abnormalities lies in detect alterations in the early stages of larval development that can subsequently affect the expression of a normal phenotype, those being periods of great vulnerability to changes in the environment. Also, must be considered that anuran larvae have been proposed as experimental models for assessing teratogenic effects because of the tadpole's phylogenetic proximity and similarity in embryogenesis to the rest of the vertebrates, including human beings.

## 2. Materials and methods

### 2.1. Study area

In the study area—it located on the outskirts of the city of La Plata (Buenos Aires, Argentina)—two zones were selected having different types and magnitudes of agrarian activity. Reference zone 1 (Z1), the location of sampling Site 0 (S0), lies southeast of the city, in the central region of the floodplain of the El Pescado Stream (Fig. 1, southeast portion and the lower *inset*). S0 contains mainly grasslands with a low degree of anthropic influence and currently with livestock. In the topographically lower lying sectors, temporary ponds are formed. Records of agricultural management during the past 15 years and measurements of the quality of the water and sediment are available (Natale, 2006, Peluso, 2011). Zone 2 (Z2; Fig. 1, northwest portion and upper *inset*) is located within the PGB due West of the city of La Plata and lying in the upper portion of the basin of the Carnaval Stream. Records on the management of this field during the last 10 years along with measurements of the sediment at the study sites during the more recent years are likewise available (Mac Loughlin et al., 2017). This area receives a high anthropic impact in the form of intensive cultivation under cover. Five sites (S1 through S5) with geomorphologic characteristics similar to S0 were selected.

### 2.2. Sampling

Whole sediment samples were taken according to methodologies standardized by the American Society of Testing Materials (ASTM, 2002) at all the selected sites during a fallow period (in order to discard possible effects of acute pulses of contamination like drift and runoff). Each composite sample was constituted from at least 10 discrete grab samples per site, as recommended by the ASTM protocol. A stainless-steel blade was used for the extraction of sediment in portions of 10 $cm^2$

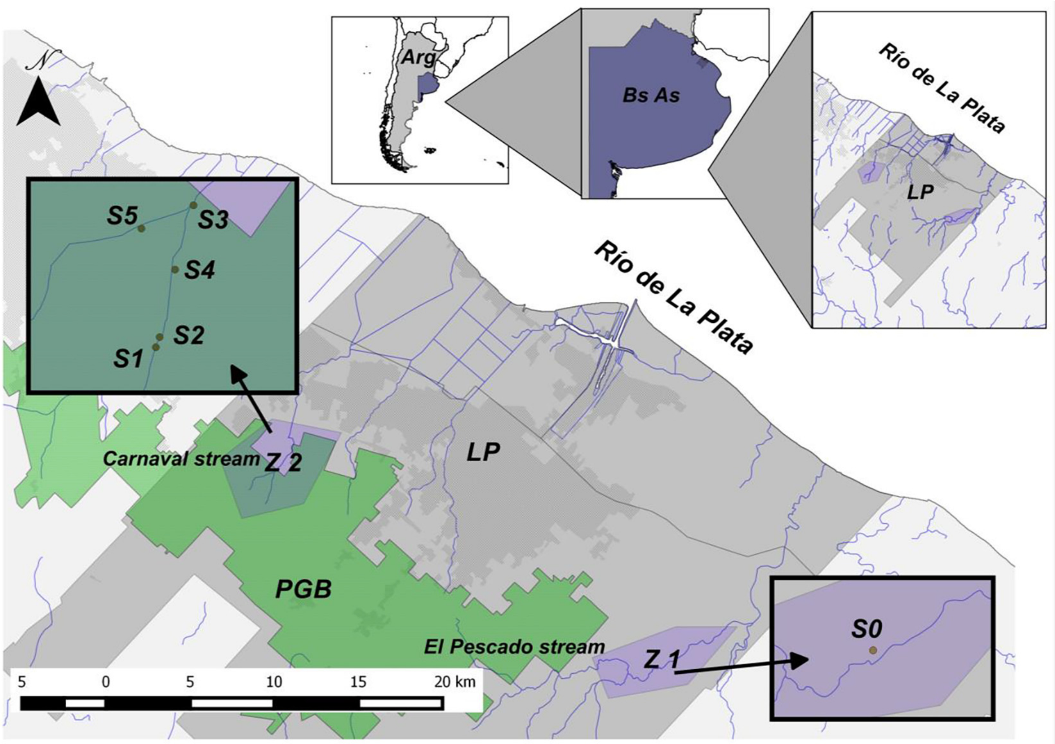

**Fig. 1.** Study area. Arg: Argentina; Bs As: Buenos Aires; LP: La Plata; Reference Zone 1 (Z1), the location of site 0 (S0), and Zone 2 (Z2) within the periurban green belt (PGB) with intensive cultivation under cover, the location of five sites (S1 through S5).

within the first 5 cm of depth. The sediments were placed in plastic bags for transfer to the laboratory. The fraction corresponding to the bioassay of toxicity was preserved at 4 °C in the dark until testing (Peluso et al., 2013a), while the fraction for chemical analysis was stored at –20 °C until use.

### 2.3. Species used in the study

*Hc* individuals and *Bp* clutches were collected from sampling Site S0, placed in plastic containers with water from the same sites, and transferred to the laboratory. In accordance with the methodology proposed for *H. azteca* (Call et al., 1998), the amphipods were cultured for several generations in dechlorinated water at 20 ± 1 °C with constant aeration and a 16:8-h light:dark photoperiod. Since *Bp* lay a large number of eggs per clutch we selected a small portion of different clutches in order to guarantee the collection of a representative sample. Clutch samples were composed of 10% of each of three different clutches with conservation purposes (Collection permit: 22500–33443/16). The fertilized eggs were placed together in 45-L tanks, at a density of 10 individuals/L, with aeration and dechlorinated water at 25 ± 1 °C and maintained in the same light–dark cycle in accordance with the methodology proposed by Natale et al. (2018).

### 2.4. Toxicity bioassays

The bioassays with amphipods were conducted following standardized protocols for *H. azteca* (USEPA, 2000) along with the recommendations suggested by Peluso et al. (2011) for *Hc*; while those with the anurans were conducted following standardized protocols for *Rana pipiens* (ASTM, 2007), with minor modifications for *Bp* including a higher temperature, less frequent water renewal and continuous aeration according to aquatic bioassays with *Bp* (Natale, 2006, Natale et al., 2006, Natale et al., 2018). Preliminary tests were carried out for both species under the conditions summarized in Table 1. The procedure stated in brief: Each experimental unit (*i.e.*, test chamber) consisted in 100 ml of whole sediment plus 175 ml of dechlorinated water. The samples were left standing for 24 h according to the protocols for both species, until the physicochemical conditions had become stabilized. The conditions used were considered to be representative of those established in the field with respect to the exchange between the sediment and water phases. For this reason, the physicochemical measurements in the laboratory were performed after that point. The hardness, alkalinity, conductivity, and pH of the water were measured in each test chamber at the beginning and end of the test and the temperature and dissolved oxygen daily; with the readings of dissolved oxygen, conductivity, pH, and temperature made with a multiparameter model WA-2017SD. The hardness was determined by titration with EDTA of the calcium present according to Method 2340C, and alkalinity by acid-base titration according to the method 2320 (APHA, 1998). A control of water with no sediment was included. Note that all individuals were inspected under a binocular stereoscope before exposure in order to discard any alteration.

### 2.5. Ecotoxicologic endpoints evaluated

Mortality was determined for both species every 24 h by direct observation following the criteria described for *Hc* (Peluso et al., 2011) and *Bp* (Natale et al., 2006, Pérez-Iglesias et al., 2014). At the end of the bioassay (10 days), all individuals were fixed in 10% (v/v) aqueous formaldehyde for further evaluation. Growth was determined in both species by measuring the length of the body with a digital caliper to the nearest 0.01 mm in accordance with descriptions made for *Hc* (Peluso et al., 2011) and *Bp* (*i. e.*, snout-to-vent length; Natale, 2006). The endpoints previously used for *Bp* were supplemented with measurements of behavior, development, and morphologic abnormalities since those characteristics are appropriate indicators of sublethal effects. Alterations in swimming were recorded every 24 h after gently swirling the water five times with a glass rod and observing the natatory characteristics of each individual tadpole for 1 min. Irregular swimming (IS) and immobility (IM) were classified and scored according to the

**Table 1**
Conditions of the tests performed with the amphipod *Hyalella curvispina* (*Hc*) and the anuran tadpole *Boana pulchella* (*Bp*).

| Test conditions | *Hc* | *Bp* |
|---|---|---|
| Type of bioassay | full sediment toxicity test | |
| Duration | 10 days | 10 days |
| Room temperature | 21 ± 1 °C | 25 ± 1 °C |
| Photoperiod | 16L:8D | |
| Light quality | fluorescent light, 100–1000 lux | |
| Test chambers | glass, 500 ml | glass, 1000 ml |
| Replicates | 4 | 6 |
| Sediment volume | 100 ml | |
| Water volume | 175 ml | |
| Water renewal | partial, every 24 h | partial, every 48 h |
| Water quality | hardness = 115 ± 33 mg $CaCO_3$/L; alkalinity = 115 ± 5; dissolved oxygen = 7.0 ± 1.0 mg/L; conductivity = 1.16 ± 0.08 mS/cm; pH = 8.1 ± 0.2; temperature = 21 ± 1 °C | |
| Organisms per chamber | 10 | 5 |
| Age | 7–14 days | 10 days |
| Stage of development | Juvenile | Gosner stage 25 |
| Feeding | lettuce, every three days | Tetramin® flakes, daily |
| Aeration | no aeration | continuous |
| Endpoints | mortality and growth | mortality, growth development, behavior, morphologic abnormalities |
| Acceptability | minimum average survival of over 80% | |

descriptions made by Brunelli et al. (2009) and the modifications introduced by Pérez-Iglesias et al. (2015). The inhibition of development and the presence of abnormalities were assessed in individuals by visual observation under an Ahecro ZTX-3EI stereoscopic binocular microscope. The stage of development was determined according to the classification proposed by Gosner (1960). Abnormalities were at first classified after the categories proposed by Bantle et al. (1996), but were then modified to obtain variables that enabled a rigorous statistical analysis distinguishing not only the type but also the degree of the effect (*i.e.*, low, medium, or high severity) considering the ability of a) escaping from a predator, b) feeding normally, and c) reaching metamorphosis —*e.g.*, tail flexure (TF, a bending generated from the bottom or in the middle region of the tail by moving it side or vertically), hypopigmentation (HP, a marked depigmentation of the body and tail resulting from the reduced population of melanophores), displaced intestine (DI, an abnormal bowel, without the normal spiral shape), absence of keratodonts (AK, a significant lack of keratodonts of at least ten consecutive pieces, in one or more rows), subcutaneous air (SA, the abnormal presence of one or more air bubbles in the visceral cavity, on either or both sides of the body).

### 2.6. Chemical analysis

The content of organic matter in sediments was determined by calcination (% of weight loss on ignition, % LOI) at 550 °C (APHA, 1998, Heiri et al., 2001). We selected representative molecules like persistent organochlorine compounds and pesticides of different families. The analytical determination of pesticides was carried out through an extraction involving the modification of a Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) procedure: 7 g of wet sediment were extracted with 15 ml of acetonitrile, 2 g of NaCl, and 6 g of anhydrous $MgSO_4$. An analysis was then performed by a combination of gas chromatography linked in tandem to time-of-flight mass spectrometry. The conditions of extraction, instrumentation, total-pesticide determination, and limits of detection and quantification are detailed in Mac Loughlin et al. (2017).

### 2.7. Statistical analysis

All the statistical analyses were performed according to Zar (2013). The data analysis was conducted with R software version 3.1.1 (R Development Core Team, 2014). The level of significance was set at 0.05 for all tests. In order to characterize the multivariate profile of each site in relation to the variables directly associated with sediments (pesticides + LOI), a principal-component analysis (PCA) was performed. The censored data were treated according to the criteria of Helsel (2012). The analysis generated an ordering of sites depending on the physicochemical matrix. Likewise, the variables in the condition of the test water were projected without actively participating in the analysis. The mortality and growth endpoints were also projected in the PCA in order to evaluate the information provided by those bioassays (Husson et al., 2017).

The proportion of individuals affected per test chamber was calculated for the lethal (mortality) and sublethal endpoints (growth and behavior). Each proportion was angular-transformed and a one-way ANOVA with Dunnett's *post hoc* test performed in order to determine significant differences from the reference group (S0). The normality and homoscedasticity were corroborated by the Shapiro-Wilk and Bartlett tests, respectively. The stages of development were compared by means of the Kruskal-Wallis nonparametric analysis with a subsequent test of multiple comparisons. The frequency of abnormalities was analyzed in several ways: i) by a contingency table (5 rows by 6 columns) to determine the independence of different abnormalities (n = 5) and sites (n = 6); ii) by a chi-square goodness-of-fit test performed to determine if the observed frequencies of abnormalities were uniformly distributed among the treatments; iii) a Kruskal-Wallis analysis with a subsequent test of multiple comparisons to determine if significant differences existed between sites; and iv) a multiple-correspondence analysis (MCA) to determine the association between the sites and the abnormalities studied and make a comprehensive interpretation of the information provided by those variables. Thereafter the sites were projected to characterize the profile of abnormalities in each one (Husson et al., 2017).

## 3. Results and discussion

### 3.1. Physicochemical analysis

Sediments constitute an environmental compartment that acts as a sink (*i.e.*, a final destination) for pollutants (Burton and Landrum, 2003). Especially pesticides with a high octanol-water–partition coefficient (Kow) adhere to suspended particulate material and are then incorporated into the bulk sediment (Linders and Luttik, 1995). Different pesticides that had been employed in the area were detected in the sediments of the portion of the Carnaval Stream that crosses Z2 at a particular time of the agricultural cycle (*i. e.*, the fallow period). The sediment in this area is characterized by clayey mud (Ronco et al., 2007). The compounds detected were two herbicides, trifluralin and acetochlor, and three insecticides, chlorpyrifos, endosulfan, and lambda-cyhalothrin. The data obtained in the present study were consistent with the presence of herbicides and insecticides that had been previously reported in the area (Jergentz et al., 2005, Marino and Ronco, 2005, Agostini, 2013, Mac Loughlin et al., 2017). Specifically, the presence of Acetochlor, Atrazine, Trifluralin, Pendimethalin, Bifenthrin, Cypermethrin, Deltamethrin, Permethrin, Lambda-cyhalothrin, Chlorpyrifos, Diazinon, Malathion, Parathion, Methyl parathion, Fipronil, Endosulfanes, α-Lindane, β-Lindane, γ-Lindane, Heptachlor, Heptachlor epoxide (isomer A), Heptachlor epoxide (isomer B), o,p′-DDE, p,p′-DDE, Dieldrin, p′p-DDD, Endrin, o,p′-DDT, p,p′-DDT, Aldrin, Pyraclostrobin, Azoxystrobin, Epoxiconazole, and Tebuconazole was determined.

Table 2 summarizes the physicochemical matrix, listing the parameters measured and pesticides detected in the test chambers. Fig. 2 presents the results of the PCA performed in order to classify the environmental quality of the sites on the basis of the quality of the sediments. The first dimension explains more than 40% of the variability of the data and generates an order from left to right, as the number of pesticides increases above the limits of detection. The second dimension accounts for almost 30% of the variability and indicates mainly a separation on the basis of the presence of endosulfan. The profile of the sites and the quality of the sediments could be categorized into the following five groups: i) S2 with a high number of pesticides detected but with the absence of endosulfan, ii) S4 with a high number of pesticides detected including endosulfan, iii) S5 with a low number of pesticides detected including endosulfan, iv) S1 and S3 characterized by a low number of pesticides detected along with the absence of endosulfan, and finally v) S0 with no detected pesticides at all. This last group, exemplified by the sampling site therein, is thus characterized by an increase in the LOI values in the sediment samples at SO. This analysis enabled an ordering of the sites according to the presence of pesticides as follows: $S2 = S4 > S5 = S1 > S3 > S0$ (Fig. 2). These results are consistent with those reported by Mac Loughlin et al. (2017) for the same sites. Since S4 and S5 are located downstream from S2 (*cf.* Fig. 1, upper left *inset*), we expected that the former two sites would have intermediate environmental conditions between those of S2 and S3. The absence of pesticides in the reference site S0 coincides with previously reported results (Natale, 2006, Demetrio, 2012).

### 3.2. Biological analysis

#### 3.2.1. Mortality and growth inhibition

The control of water with no sediment exhibited a total absence of any alteration. The average survival under the conditions of the reference site (S0) was 93.5% for *Hc* and 100% for *Bp*, thus conforming with the acceptance criteria recommended for the bulk-sediment test. The dissolved oxygen in the overlying water was at or above acceptable levels (*i.e.*, 2.5 mg/L) during all the treatments throughout the study (USEPA, 2000, OECD, 2004, ASTM, 2007).

The comparison of the endpoints evaluated by the PCA (Fig. 2) demonstrated that the mortality and growth of each species were situated in the same quadrant. The results with the mortality endpoint indicated significant differences ($F[5,18] = 11.500$, $p < 0.001$), but only between the sediment samples from S0 and S2 for *Hc*. No such significant differences were detected for *Bp* ($F[5,29] = 0.896$, $p = 0.497$), with this absence of mortality being in agreement with results previously reported in similar systems for anuran tadpoles (Snodgrass et al., 2008, Brand et al., 2010, Peltzer et al., 2013). In view of those combined data, we concluded that anuran larvae were not highly sensitive to the environmental status of sediment samples with respect to the endpoint of mortality; but lethality had occurred upon exposure of anurans from embryonic stages to similar sediment samples (Fort et al., 1999, Brand et al., 2010).

The different sublethal endpoints evaluated revealed significant effects among the sites. The sediment samples from S3, S4, and S5 caused a significant inhibition of growth in *Hc* ($F[3,117] = 10.142$, $p < 0.005$) compared to that occurring with the S0 sediment. These results coincide with those reported by Mac Loughlin et al. (2017), who also observed a growth inhibition in *Hc* exposed to sediments having a profile of pesticides similar to that described in this work. Moreover, Peluso et al. (2013a) found comparable results for this species in the sediments of rivers and streams from the pampas region containing endosulfan and pyrethroids. These data demonstrate the sensitivity and reproducibility of the bioassay with *Hc* for the assessment of sediment toxicity. In addition, the larvae of *Bp* evidenced a significant growth inhibition ($F[5,167] = 2.460$, $p < 0.05$) when exposed to sediments from S2, which effect may be attributable to the chemical profile of that particular site (*i.e.*, a high number of pesticides) that at the same time resulted in mortality in *Hc* (Fig. 2). An inhibition of growth in tadpoles had been previously reported in similar systems (Peltzer et al., 2013). Additionally, Fig. 3 showed the comparison of the sensitivity of *Hc* and *Bp* considering mortality and growth inhibition. The amphipod turned out to be the more sensitive to the chemical profile of the agroecosystem.

#### 3.2.2. Complementary endpoints in anuran tadpoles

With respect to behavior and the stage of development, no significant differences were found among the individuals exposed to the sediments from Z1 and Z2 ($F[5,29] = 1.539$, $p = 0.208$; H[5,

**Table 2**

Physicochemical matrix with respect to different parameters and pesticides detected.[*]

| Site | Hardness | Alkalinity | Dissolved oxygen | Conductivity | pH | Temperature | LOI | Trifluralin | Acetochlor | Chlorpyrifos | Endosulfan | L-Cyhalothrin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 100 | 100 | 6.4 | 0.63 | 6.1 | 22.1 | 28.0 | – | – | – | – | – |
| 1 | 184 | 165 | 5.8 | 0.94 | 7.5 | 21.4 | 15.0 | 2.1 | 2.7 | – | – | – |
| 2 | 152 | 85 | 5.6 | 0.98 | 7.6 | 21.2 | 7.8 | 55.9 | 17.7 | 26.0 | – | 265.0 |
| 3 | 132 | 80 | 5.3 | 0.94 | 7.6 | 22.1 | 3.4 | 6.2 | – | – | – | – |
| 4 | 128 | 95 | 5.6 | 1.01 | 7.8 | 22.2 | 4.7 | – | 1.1 | 9.0 | 16.6 | 48.6 |
| 5 | 183 | 130 | 5.0 | 1.06 | 7.8 | 21.6 | 3.5 | – | dnc | – | 12.0 | – |

[*] The hardness and alkalinity are expressed in mg $CaCO_3$/L, the dissolved oxygen in mg/L, the conductivity in μSiemens/m, the temperature in °C, and the pesticide concentrations in μg/kg dry weight. LOI, % of weight loss on ignition; dnc, detected but not quantified.

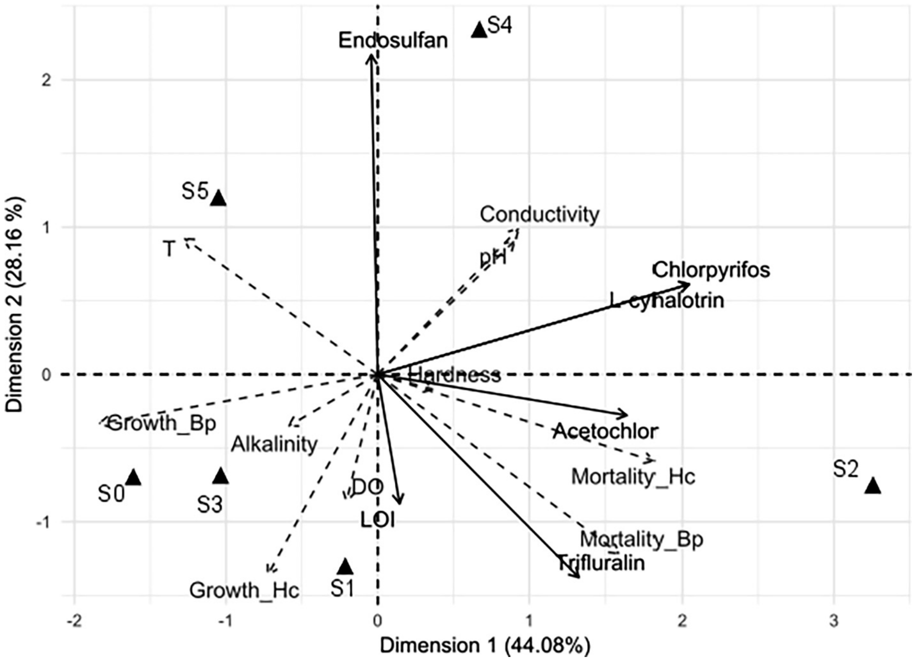

**Fig. 2.** Principal-component analysis performed to characterize the multivariate profile of each site (S0–S5) as a function of the variables directly associated with the sediments (*i. e.*, pesticides + weight loss on ignition) plus the additional variables associated with the water of the test conditions (*i. e.*, conductivity, pH, hardness, and alkalinity). The variables of the mortality and growth of the two species are projected onto the plane for association with the profile of the pesticides. S0, grasslands with a low degree of anthropic influence; S1–S5, sites in areas with a high degree of anthropic influence; Hc, *Hyalella curvispina*; Bp, *Boana pulchella*; LOI, weight loss on ignition; DO, dissolved oxygen.

173] = 10.121, p = 0.120, respectively). Five types of morphologic abnormalities, however, were detected. The analysis of the occurrence of abnormalities accordingly indicated that 28.3% of the 180 individuals exposed had highly significant frequencies of abnormalities in the following decreasing order (Fig. 4, Table 3): HP (12.2%) > AK (9.4%) > SA (3%) > TF (2.7%) > DI (0.5%). The most frequent occurrences were recorded in the tadpoles exposed to the sediments from S4, which site contained several pesticides.

The relevance of evaluating abnormalities in detail lies in detect alterations in the early stages of larval development, those being periods of great vulnerability to changes in the environment, since morphogenesis happens, and the *Bauplan*, or scheme of bodily development, along with the correct functioning of the organs and physiologic systems, are determined in an individual at that time (Cock, 1966).

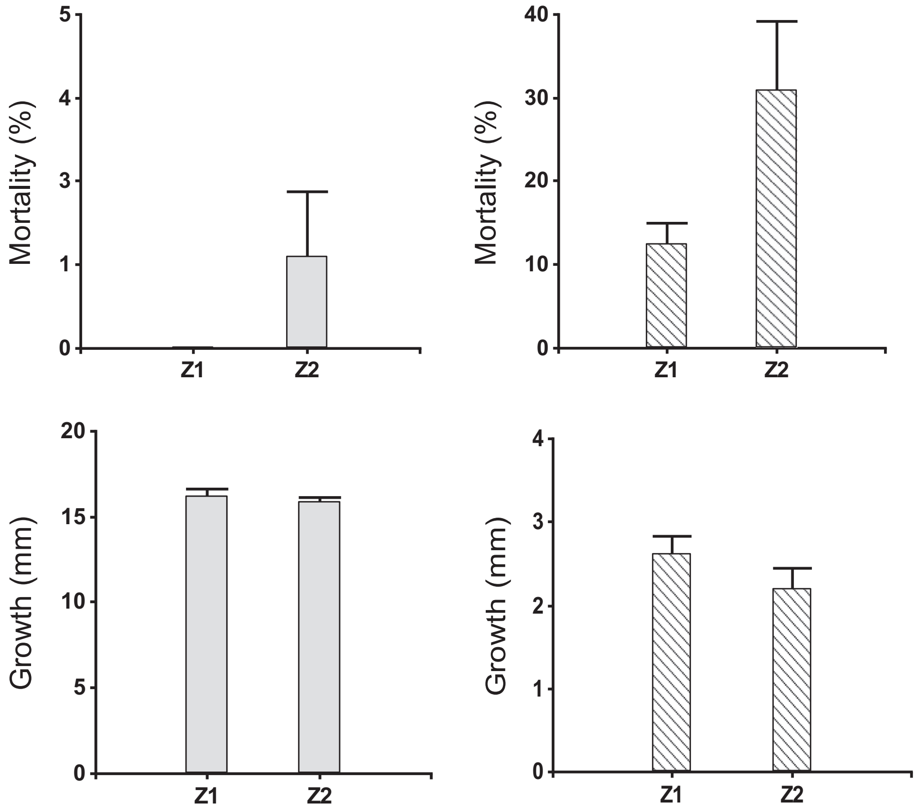

**Fig. 3.** Comparison of the sensitivity of *Hyalella curvispina* (striped box) and *Boana pulchella* (grey box) considering mortality and growth inhibition (in millimeters). Provided values are mean ± standard error per zone. Z1: La Plata Reference Zone 1; Z2: Zone 2 with intensive cultivation under cover.

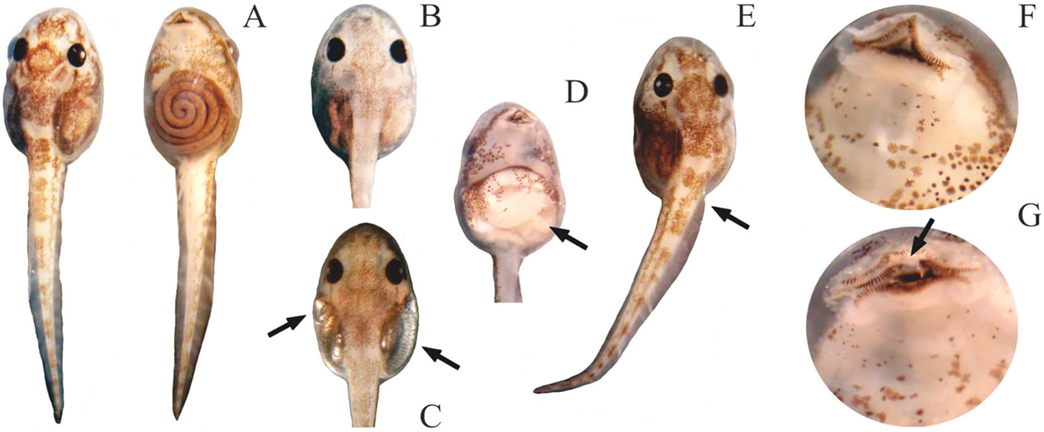

**Fig. 4.** Morphologic abnormalities detected in tadpoles of *Boana pulchella*. Panel A, normal tadpole without morphologic abnormalities; Panel B, hypopigmentation; Panel C, presence of subcutaneous air (arrows); Panel D, displaced intestine (arrow); Panel E, tail flexion (arrow); Panel F, normal keratodonts; Panel G, absence of keratodonts (arrow).

**Table 3**
Relative frequency (in percentage) of detected abnormalities according to each site (S).[*]

| Sites | TF | HP | DI | AK | SA | TOTAL |
|---|---|---|---|---|---|---|
| S0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S1 | 1.7 | 0 | 0 | 0 | 0 | 1.7 |
| S2 | 1.1 | 0 | 0 | 2.2 | 0 | 3.3 |
| S3 | 0 | 1.7 | 0.6 | 0.6 | 0 | 2.8 |
| S4 | 0 | 8.9 | 0 | 1.1 | 0 | 10.0 |
| S5 | 0 | 1.7 | 0 | 5.6 | 3.3 | 10.6 |
| TOTAL | 2.7 | 12.2 | 0.6 | 9.4 | 3.3 | 28.3 |

[*] Tail flexure (TF); hypopigmentation (HP); displaced intestine (DI); absence of keratodonts (AK); presence of subcutaneous air (SA).

Therefore, any significant influence that can potentially cause changes during early development can subsequently affect the expression of a normal phenotype (Gans, 1985). The scheme of embryologic development provides a means of deducing and explaining the mechanisms by which such alterations produce the deleterious effects suffered by exposed individuals (Brennan, 1991). We need to stress that anuran larvae have been proposed as experimental models for assessing teratogenic effects because of the tadpole's phylogenetic proximity and similarity in embryogenesis to the rest of the vertebrates, including human beings (Bantle et al., 1996, Schmitt et al., 2014).

Among the abnormalities recorded, HP was clearly identified because of that alteration's relevance to and consequences for normal development, as reported by Brunelli et al. (2009). HP was mostly observed in individuals exposed to the sediments from S4, with those being characterized by contamination with endosulfan. We considered that alteration as an abnormality of medium severity since the decrease in melanophore expansion could represent a loss in the ability of larvae to remain hidden from predators and could also make those individuals more sensitive to ultraviolet irradiation. Nevertheless, we consider that individuals with HP would be able to pass through the stages of metamorphosis without difficulty.

The absence of keratodonts was mostly observed in individuals exposed to the sediments from S5, those also being contaminated with endosulfan. An effect on oral structures, especially AK, has likewise been reported upon exposure to heavy metals and pesticides (Rowe et al., 1998, Medina et al., 2013, Babini et al., 2015, Bach et al., 2016). This abnormality was similarly considered of low severity since individuals still could feed normally. However, it is known that this absence could also be influenced by other ingested items like food or sediment. Furthermore, the incidence of oral deformities in association with fungal infection may be high in natural populations, with those types of mycoses having a strong influence on the occurrence of such alterations in tadpoles (Drake et al., 2007).

The majority of the individuals who exhibited TF had been exposed to sediments from S1. This abnormality had been reported in several publications and for several pollutants, such as heavy metals (Pérez-Coll et al., 1988, Haywood et al., 2004) and pesticides (Brunelli et al., 2009, Agostini et al., 2010, Svartz et al., 2016b, Svartz et al., 2016a). Thus, the alteration of TF is likely not to be a specific ecotoxicological endpoint. We therefore classified TF as an abnormality of medium severity since that alteration can nevertheless cause the individual to move with difficulty and thus become an easy prey as well as be less able to feed. Nonetheless, we consider that TF in the tadpole will not cause later problems in the postmetamorphic stages of development.

The tadpoles that were found with SA were observed in sediments from S5, with those as well containing endosulfan. This abnormality had not been previously registered in the scientific literature. We described several small bubbles on the sides of each tadpole body; which inclusions, being located exclusively in the subcutaneous cavity, did diminish the tadpoles mobility. We thus classified SA as an abnormality of medium severity.

Finally, because individuals who exhibited DI were very few, we could not attribute that abnormality to any particular sediment. The degree of severity must still be confirmed depending on the survival of the affected individuals.

On the basis of the quantification and frequency of occurrence of these abnormalities we performed uni- and multivariate analyses in order to evaluate the information from each relevant set of variables. The contingency table analysis demonstrated the absence of an independence between these abnormalities and each of the various sites ($\chi^2 = 104.13$, df = 25, $p < 0.05$). The chi-square goodness-of-fit test indicated that not all abnormalities were uniformly distributed ($\chi^2 = 32.30$, df = 5, $p < 0.05$) except for ID ($\chi^2 = 2.90$, df = 5, $p = 0.732$). The Kruskal-Wallis test enabled a detection of any significant differences between the frequency of each abnormality and the sites, in corroboration of the results with the MCA. Fig. 5 presents the results of the MCA performed to evaluate the association between the sediments of the sampling sites and the morphologic abnormalities and to characterize those sites on the basis of the occurrence of such aberrations. According to this analysis, the profile of abnormalities and sites could be categorized in four significantly different groups ($p < 0.05$) characterized by: i) a low frequency of abnormalities in association with S0, S2, and S3; ii) a high frequency of individuals with TF in association with S1; iii) a high frequency of individuals with HP in association with S4; and iv) a high frequency of individuals with SA and

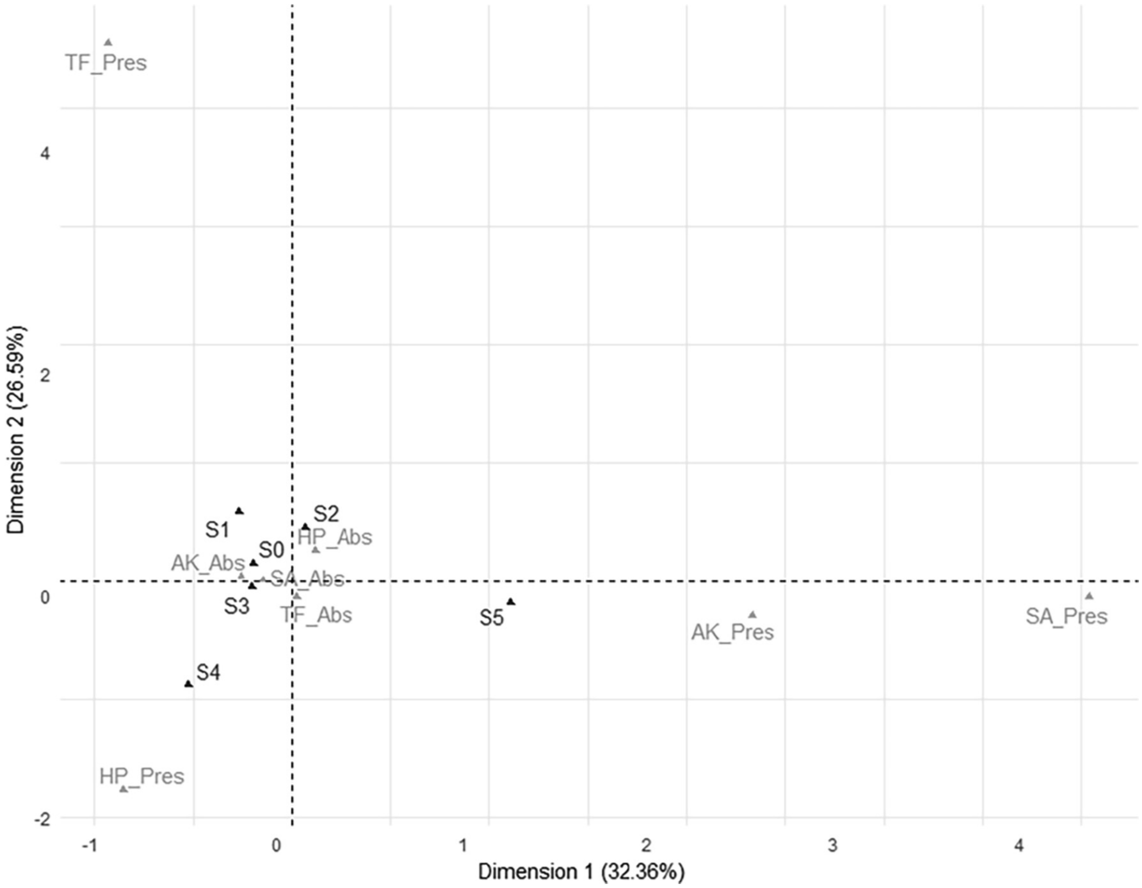

**Fig. 5.** Multiple-correspondence analysis generated by the presence (Pres) or absence (Abs) of morphologic abnormalities detected in tadpoles of *Boana pulchella*. The sites were chosen to observe associations between the sediments in those environments and the profile of possible abnormalities. S0, grasslands with a low degree of anthropic influence; S1–S5, sites in areas with a high degree of anthropic impact; TF, tail flexion; HP, hypopigmentation; SA, presence of subcutaneous air; AK, absence of keratodonts.

AK in association with S5. Therefore, we concluded that the high frequency of abnormalities coincided specifically with the presence of endosulfan, as had been reported by Brunelli et al. (2009), which compound is an organochlorine insecticide with a high persistence in the environment.

### *3.3. Relevance of the experimental approach*

The information obtained on the basis of only biologic variables (Fig. 2) indicated the results summarized as follows: (i) Both species (*Hc* and *Bp*) were useful for diagnosing the study areas and characterizing the sediments at the sites according to the following decreasing order of toxicity: S2 > S4 = S5 > S3 > S1 > S0. (ii) The sediment at Site S2 produced mortality in *Hc* and a growth inhibition in *Bp*. (iii) The sediments at Sites S4 and S5 caused a growth inhibition in *Hc* and morphologic abnormalities in *Bp*. (iv) The sediment at Site S3 resulted in a growth inhibition in only *Hc* and low frequency of abnormalities in *Bp*. Finally, (v) the sediments at Site S1 showed low frequency of abnormalities in *Bp* and the sediments at S0 were totally devoid of any adverse ecotoxicologic effects. In the comparison of the information generated by the chemical and biological analyses, both forms of evaluation—*i.e.*, the PCA and the MCA—agree on the overall characterization of the environments under investigation (Figs. 2 and 5). According to a recent survey of the use of agrochemicals in the province of Buenos Aires, 168 biologically active compounds of different toxicologic categories had been applied in Z2. As a result, that region had the highest value in the consequent index of horticultural danger based on the amount of active ingredients used for each production, the hazard involved to the biota, and the area cultivated, among other considerations (Defensor del Pueblo, 2015).

Toxicity bioassays and the chemical characterization of environmental samples of sediments are two essential lines of evidence in assessing and categorizing the quality of polluted water bodies (Abessa et al., 2008). Amphipods—and in particular the genus *Hyalella*—are extensively used in evaluations of systems polluted with pesticides since those crustaceans are organisms with a high sensitivity to toxic agents, and especially to organochlorine and pyrethroid insecticides (Li and You, 2015). Although studies assessing the sensitivity of the native amphipod *Hc* with respect to the pollutants detected in the study area of the present investigation are scarce, the results presented here coincide with previous reports dealing with the sensitivity of this species and the reproducibility of its use for the assessment of sediment quality (Peluso, 2011, Peluso et al., 2011). Therefore, *Hc* as an indicator was found to respond accurately despite variations related to the management of the crops within the study area. Thus, even though *Hc* has been found to be useful in the present laboratory bioassays, such native species must be incorporated into additional studies of sediment quality and the results evaluated.

In view of the above considerations, and although anurans are good indicators of environmental conditions (Simon et al., 2011), up to the present time and the research reported here, only few studies have been undertaken employing anuran larvae or embryos to evaluate the toxicity of both natural (Fort et al., 1999, Snodgrass et al., 2008, Brand et al., 2010, Peltzer et al., 2013) and artificial (Francis et al., 1984) sediments. The present work is furthermore the first to use anuran larvae of a native species (*Bp*) to evaluate the toxicity of sediments from an agroecosystem as well as being the first to compare the responses of this species to those of *Hc*, the latter species having been already validated as a biologic indicator for the assessment of sediment toxicity. Consequently, we intend to incorporate *Bp*—it being furthermore a vertebrate species—into sediment testing since that anuran is representative of the pampean region and is naturally present in these local agroecosystems (Cei, 1980). Finally, the utility of that species in toxicity bioassays has been corroborated by numerous ecotoxicological studies.

We need to reemphasize that sediments are the final destination of many pollutants and indicators of the local and historical pollution of aquatic ecosystems. Although bioassays with that environmental compartment have constituted a widely useful, simple, and economical sample for diagnosing pollution, very few studies have been undertaken employing that approach in the rivers and streams of developing countries. In this regard, the present results have demonstrated that bioassays involving both species constitute a promising tool in programs aimed at the management and protection of freshwater bodies.

## 4. Conclusions

1. Bioassays with sediments enabled a characterization of the environmental quality of the areas in the present investigation.
2. The species *Hc* proved to be the more sensitive than *Bp*. In view of the chemical profile of the agroecosystem under study—it dominated by insecticides designed to kill arthropods with a certain margin of safety for vertebrate species—the results were nevertheless highly consistent.
3. *Bp* is suitable for performing bioassays with whole sediment and is considered a promising model for ecotoxicological studies or the diagnosis of environmental quality. The species provided additional information that was both complementary and significant in view of the species's phylogenetic proximity and similarity in embryogenesis to the rest of the vertebrates, including *Homo sapiens*. An analysis of the occurrence of morphologic abnormalities enabled the detection of toxicity in sediments not affecting other endpoints.
4. We wish to emphasize the fundamental relevance of working with native species from different taxonomic levels in order to provide complementary information in the diagnosis of an environment. The study reported here contributes useful information for application to management programs involving the environmental control of regional water bodies. In conclusion, both the species *Hc* and *Bp* should be considered appropriate as tests organisms for the assessment of sediment quality as well as effective and promising environmental indicators.

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