TADPOLES ASSAY: ITS APPLICATION TO A WATER TOXICITY ASSESSMENT OF A POLLUTED URBAN RIVER

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Abstract. The acute toxicity assay with premetamorphic tadpoles of *Bufo arenarum* as sentinel organism was applied to evaluate the quality of two water samples taken from three sites of the Reconquista River, an urban watercourse which is recipient of both untreated industrial effluents and domestic wastes. The results of the 96 hr bioassays were compared with the physicochemical parameters determined in the samples. Mortality rates in each sample were compared using the Bonferroni's test and a stepwise regression analysis of mortality and physicochemical parameters was done. In this way, it was possible to build up consistent descriptive models which showed that pH, Cl^-, Cd^{2+} and Cu^{2+} concentrations in the river water were significant independent variables and might explain, under the experimental conditions, the recorded toxicity effects of the tested samples. Because of its simplicity, low cost and reliability it was suggested the tadpoles bioassay be included in the set of tests used in integrated program of freshwater pollution.

Keywords: acute toxicity, aquatic toxicity, *Bufo arenarum*, Reconquista River (Argentina), river pollution, tadpoles assay

1. Introduction

Chemical parameters such as COD, BOD, OD, phenols, nitrites, heavy metals, etc. were suggested as main tools to evaluate toxicity of natural freshwater (Rand, 1995; APHA, 1995). Changes in the physical and chemical composition of a surface water may indeed alter the structure and diversity of population and communities depending on their sensitivity (Loez and Salibián, 1990; del Giorgio *et al*., 1991; Conforti *et al*., 1995).

Numerous laboratory aquatic acute bioassays were developed using species belonging to different levels in the food webs as test organisms (USEPA, 1987, 1988). The purpose of those assays was to contribute to an integral evaluation of the adverse effects of the pollutants on the freshwater biota.

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Amphibian species have shown to be more sensitive bioindicators of aquatic contaminants than other aquatic vertebrates because they have a permeable skin that readily absorbs substances from the environment (Devilles and Exbrayat, 1992; Boyer and Grue, 1995; Pierce, 1993; Schuytema and Nebeker, 1996, 1998).

The toad *Bufo arenarum* is widely distributed in South America. We have shown that its larvae are useful biological tools for performing standardized acute toxicity assessment bioassays (Ferrari, 1998). Our test has proven to be useful for the determination of lethality indexes of heavy metals and insecticides (Muiño *et al.*, 1990; Ferrari *et al.*, 1993; Salibián, 1992).

The implication of this study are twofold. First to evaluate the toxicity of the Reconquista River water using an acute bioassay technique developed in our laboratory with young tadpoles of *Bufo arenarum* and to explore the relationship between the results of the test and the physicochemical parameters of the assayed samples (Ferrari *et al.*, 1997, 1998). Because of its simplicity, low cost and reliability we suggest that the assay may be included as part of the laboratory set of tests applied on freshwater ecotoxicological monitoring programs. The second implication of this study is that our results may be applied in the design of water quality regulations.

The Reconquista River is an urban watercourse of the Buenos Aires Province, Argentina. It is 55 km long and flows through one of the most populated area of the country. It receives a complex mixture of pollutants from poorly treated or untreated domestic, agricultural and industrial sewages. Approximately three million people and some ten thousand industries are settled on its basin. Moreover, Reconquista River is used to be considered as a chracteristic example of the sustained adverse environmental impact of human activities. In this case, the contamination is provoked both from point and diffuse sources (Loez and Topalián, 1999a). We have shown that the water of the river presents a pollution gradient from upstream towards its mouth (Castañé *et al.*, 1998a, b); in addition each point of the river presents typical algal communities (Loez and Salibián, 1990; Loez *et al.*, 1998). A preliminary report of the results here presented was published elsewhere (Salibián *et al.*, 1998).

2. Materials and Methods

Surface water samples were collected using a pump in spring (September and October). We have shown that during spring-summer season the physicochemical parameters of water indicators of toxicity increased significantly (Castañé *et al.*, 1998a, b) The three sampling points (Figure 1) were selected along to a low-to-high pollution gradient as determined in our previous works being the upstream site Cascallares (CAS) characterised as a slightly polluted point while the other two, San Martin (SM) and Bancalari (BAN) are highly polluted sites. Samples were kept at 4 °C until used.

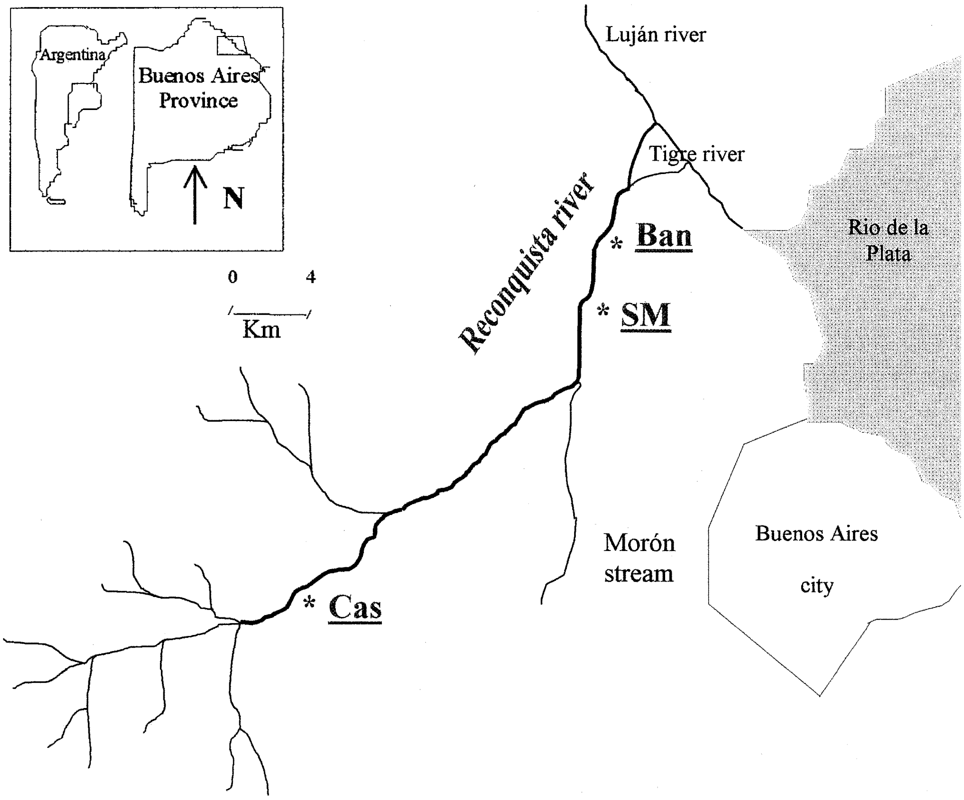


Figure 1. Geographical location of the Reconquista River, showing the location of the sampling sites.

The physicochemical parameters of the samples (Table I) were determined according to the APHA guidelines (1995) at the beginning of the assays.

Larvae at stage 26 (Gosner, 1960) were hatched in the laboratory by *in vitro* fertilization according to the procedure outlined by Castañé *et al.* (1987), which provides animals without previous contact to xenobiotics. It is to be mentioned that under our experimental conditions larvae both in control and in experimental groups stay in the same stage throughout the assays showing that the metamorphosis rate was not affected in response to their incubation media. Tadpoles were acclimated for 48 hr in US-EPA hard water (1987) of the following composition (mg L^{-1}): $NaHCO_3$, 192; $CaSO_4$, 120; $MgSO_4 \cdot 2H_2O$, 120; KCl, 8; hardness, 160–180 mg $CaCO_3$ L^{-1}; alkalinity, 110–120 mg $CaCO_3$ L^{-1}; pH, 7.6–8.0.

Cadmium chloride was used as the reference toxic at a nominal concentration of 4 ppm, which is the 96-h LC50 for premetamorphic *B. arenarum* tadpoles when incubated in US-EPA hard water (Ferrari *et al.*, 1996).

Semistatic acute tests were conducted following procedures previously described (Ferrari *et al.*, 1997). The acclimation and the assays were run at constant temperature (10 ± 1 °C) and photoperiod (12 L/12 D); animals were accommodated

TABLE I

Physicochemical parameters of surface water samples collected at three sites along the Reconquista River

	September			October			M.P.Q.
	CAS	SM	BAN	CAS	SM	BAN	
pH	9.2	7.7	7.7	7.9	7.7	7.6	
Conductivity (μS cm^{-1})	1038	1223	1507	865	1403	1240	
Hardness (mM $CaCO_3$ L^{-1})	1.3	1.8	1.8	1.2	2.05	2.55	
Alkalinity (mM $CaCO_3$ L^{-1})	11.5	10.9	11.6	9.0	11.4	11.4	
Nitrates (mg N-NO_3^{2-} L^{-1})	0.15	0.88	0.45	4.30	4.00	4.85	
Nitrites (mg N-NO_2^{2-} L^{-1})	0.03	0.04	0.04	0.19	0.05	0.05	0.06
Ammonia (mg N-NH_4^+ L^{-1})	0.15	13.70	17.10	1.70	15.80	14.20	1.37
Phosphates (mg PO_4^{3-} L^{-1})	0.3	6.0	7.4	1.8	6.25	6.15	
Chlorides (mg L^{-1})	70.5	110.0	168.0	48.5	150.0	119.5	
Phenol (mg L^{-1})	0.50	0.30	0.15	0.85	1.25	1.55	0.01
BOD_5 (mg O_2 L^{-1})	15.7	80.8	76.5	7.8	61.0	66.0	
COD (mg O_2 L^{-1})	18.0	90.3	65.3	103	300	250	
Arsenic (μg L^{-1})	30	20	20	45	15	10	50
Cadmium (μg L^{-1})	1	1	1	6	3	4	2
Chromium (μg L^{-1})	4	20	150	7	250	30	2
Copper (μg L^{-1})	70	15	40	80	45	34	0.8
Lead (μg L^{-1})	7	5	3	5	5	5	2
Zinc (μg L^{-1})	140	110	80	510	400	16	30
SPM (mg L^{-1})	171	66	47	309	226	85	

CAS: Cascallares; SM: San Martin; BAN: Bancalari; SPM: suspended particulate matter. M.P.Q.: maximum permitted quantity for freshwater life preservation considering hardness according to Argentine legislation.

at a loading rate of 1 tadpole per 10 ml (equivalent to 1 g organism/L solution) and remained unfed during the assays. Four replicates of 15 larvae each were run simultaneously. Mortality was registered every 24 hr during four days and dead organism were removed daily.

The test solutions were the following: a) hardwater control (HW); b) reference toxic control (HW + 4 ppm Cd^{2+}); c) river samples (CAS, SM, BAN); d) river samples + 4 ppm Cd^{2+} (CAS-Cd, SM-Cd and BAN-Cd); e) BAN samples diluted 1:1 with HW (BAN:HW) and f) BAN:HW + 4 ppm Cd^{2+} (BAN:HW-Cd). Assays in solutions e) and f) were conducted to search the recovery capacity by dilution of a highly polluted site as BAN. All solutions were renewed daily.

Mortality rate data transformed by arcsine $(p)^{1/2}$, were analysed using multivariate analysis of variance (MANOVA) looking for significant differences ($p < 0.05$) in the mean cumulative mortality for each assay. Bonferroni's multiple comparisons test was used to evaluate intergroup response differences (Zar, 1996).

In order to search for physicochemical parameters that could describe the mortality profiles observed in each site and sample, a stepwise analysis (forward) was run. We considered the cumulative mortality every 24 hr as the dependent variable and the physicochemical parameters (in undiluted water river samples) as the independent variables (Hair *et al*., 1995). A multiple correlation among all the physicochemical parameters was performed in order to choose, in a first stage, the independent variables to be included as predictors in a descriptive equation. All tests were conducted at the 0.05 significance level.

3. Results and Discussion

The cumulative mortality of tadpoles is shown in Figures 2 and 3; differences between groups are indicated in Table II.

Reference cadmium solutions showed 53% mortality at the end of the bioassays, indicating similar sensitivity conditions of animals in both assays (Figures 2b and 3b).

It is clear from the multiway analysis that there is a difference in the mean cumulative mortality from both bioassays. Neither in September nor in October mortality of tadpoles belonging to the control HW or the CAS group (the upstream sampling site) was observed. Therefore, it was tempting to speculate that the statistical dissimilarities obtained might be related to different water toxicities registered in the SM and BAN groups.

In order to evaluate the differences among groups, the results of the assays were analysed separately, the correlation of the physicochemical parameters with the results that came out from the bioassays was determined analysing together both sets of data.

The results of the Bonferroni's test (Table II) revealed significant differences between samples regarding larval mortality. Whereas in September BAN water

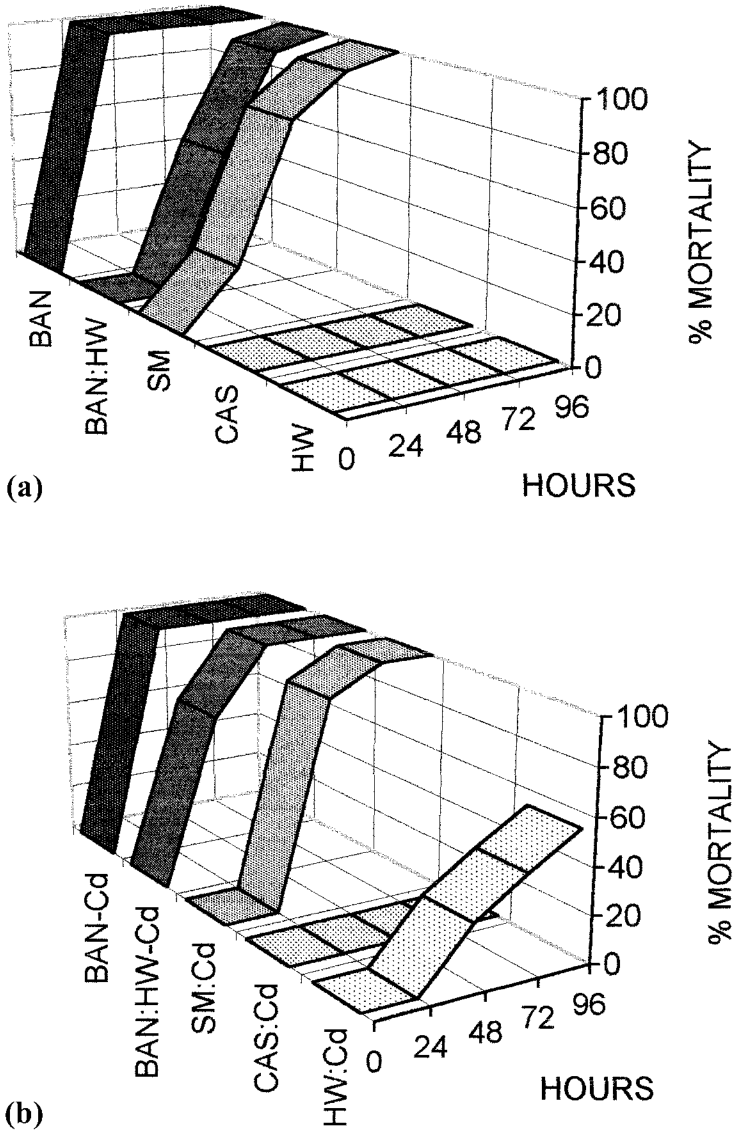
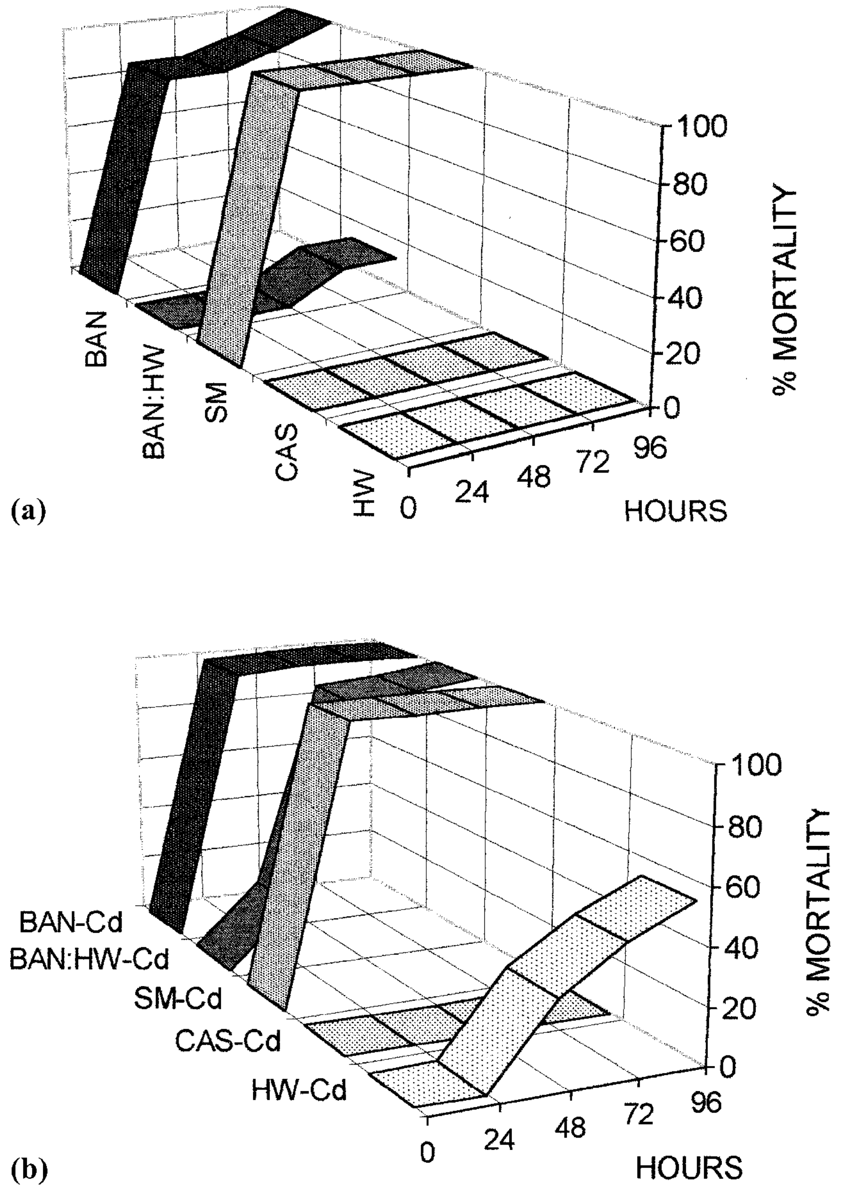


Figure 2. Cumulative mortality curves of *Bufo arenarum* tadpoles kept during 96 hr in control media and in samples of the Reconquista River (September bioassay). A: samples without Cd: BAN, BAN:HW, SM, CAS, HW. B: samples with Cd: BAN-Cd, BAN:HW-Cd, SM-Cd, CAS-Cd, HW-Cd.

was more toxic than SM water (Figure 2a), the opposite was observed in October (Figure 3a). These results could be explained as a spatial-temporal difference of water toxicity due to intermittent and punctual discharges of complex pollutant mixtures.



(a)

(b)

Figure 3. Cumulative mortality curves of *Bufo arenarum* tadpoles kept during 96 hr in control media and in samples of the reconquista River (October bioassay). A: samples without Cd: BAN, BAN:HW, SM, CAS, HW. B: samples with Cd: BAN-Cd, BAN:HW-Cd, SM-Cd, CAS-Cd, HW-Cd.

TABLE II

Multiple comparisons (Bonferroni's test) among mean cumulative mortality of tadpoles of *Bufo arenarum* kept during 96 hr in different media

Incubation media	Homogeneous groups: September assay	Homogeneous groups: October assay
HW	A	A
CAS	A	A
SM	C	D
BAN	D	D
BAN:HW	C	A
HW-Cd	B	B
CAS-Cd	A	A
SM-Cd	D	D
BAN-Cd	D	D
BAN:HW-Cd	D	C

Mortality: A = 0%, B = 20%, C = 50%, D > 90%.

Dilution of BAN water with HW decreased mortality rates in both bioassays, specially in October when its toxicity did not differ from the control HW (Table II) suggesting that the river has the potential recovery capacity by dilution.

Cadmium chloride was used as reference toxicant because it is one of the most common forms found in aqueous solutions (Stumm and Morgan, 1996) being recommended by U.S. EPA (1988). When cadmium was added, an increase in mortality was observed in both bioassays, in all groups except in the CAS group (Figures 2b and 3b). This behaviour must be a consequence of the well known fact that the toxicity of the heavy metals to aquatic organisms is dependent on the chemical form or speciation of the metal (Campbell, 1995). Furthermore, a toxicity decrease could result from a complexation of the metal with inorganic or organic constituents and by sorption onto suspended particulate matter whose values in our samples resulted considerably elevated (Table I) (Allen *et al*., 1982). It is worth mentioning that Topalián *et al*. (1999b) detected heavy metals in the Reconquista River surface water in levels always higher than the M.P.Q.; they found fluctuating changes, and postulated that this behaviour must be interpreted as an additional evidence of punctual discharges, as it was confirmed by physicochemical data.

Although in October the toxicity of the BAN-HW group was similar to the one of the control (HW), the effect of cadmium addition to the group was significantly higher than the one observed in the HW-Cd group. This fact could point out the presence of toxic substances other than the ones determined that did not make their effects evident when the sample was diluted with hardwater (HW) (Figure 3).

TABLE III

Regression equations corresponding to cumulative mortality of tadpoles and physico-chemical parameters of water samples

Mortality (P) at	Regression equation
24 hr	$P_{24} = -2.5850 + 0.0227$ [chlorides] + 0.1620 [cadmium]
	$R^2 = 0.989$, adj. $R^2 = 0.987$, d.f. = 23, F = 337.24, $p < 0.001$
48 hr	$P_{48} = 2.3952 + 0.0107$ [chlorides] – 0.3142 pH - 0.0065 [copper]
	$R^2 = 0.989$, adj. $R^2 = 0.987$, d.f. = 23, F = 342.21, $p < 0.001$
72 hr	$P_{72} = 3{,}2535 + 0.0067$ [chlorides] – 0.0158 [copper]
	$R^2 = 0.975$, adj. $R^2 = 0.964$, d.f. = 23, F = 154.07, $p < 0.001$

P: mortality; R^2: coefficient of multiple determination; adj. R^2: adjusted r-square d.f.: degrees of freedom; F: F-ratio; p: statistical significance level.

The stepwise (forward) analysis selected the variables that were included in descriptive models (Table III): 96 hr mortality was not included in the analysis because it showed all-or-none responses.

Clearly, there is no single water chemistry parameter that can explain the toxicity of the samples to the larvae, the descriptive models for each time included pH, chlorides, cadmium, and copper concentrations as significant independent variables determinants of toxicity. It is interesting to note that Cd concentrations were always close or lower than the MPQ while Cu concentration levels were 40–80 times higher; these results illustrates the importance of bioassays as a way to evaluate the integrated responses of complex mixtures of dissolved toxicants. In bioassays conducted in our laboratory with samples from the same sites of the river using juvenile fish *Cnesterodon decemmaculatus* as the test organism, the stepwise analysis also showed pH and chlorides as descriptive parameters (de la Torre *et al*., 1997; García *et al*., 1998). Descriptive model coupled to long-term monitoring programs may be useful in the design of precise predictive models for ecotoxicological monitoring programs.

The presence of a high level of chlorides and heavy metals concentrations in our samples is the consequence of untreated domestic and industrial waste discharges. Chlorides are not included in our country's law of Dangerous Residues for the freshwater life (Law No. 24051); our results suggest the convenience of adopting a maximum permitted quantity for chlorides.

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