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Field and *in Vitro* Evaluation of Ammonia Toxicity on Native Fish Species of the Central Region of Argentina

A. C. Hued,[1] M. N. Caruso,[1] D. A. Wunderlin,[2] M. A. Bistoni[1]

[1] Laboratory of Animal Diversity II, Faculty of Exact, Physical, and Natural Sciences, National University of Córdoba, Av. Vélez Sársfield 299 - 5000, Córdoba, Argentina
[2] Department of Biochemistry, Faculty of Chemical Sciences, National University of Córdoba, University City, 5000 Córdoba, Argentina

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Ammonia occurs naturally in unpolluted water bodies at low concentrations. However, high ammonia levels could be an indication of organic pollution such as from domestic sewage, industrial waste and fertilizer run-off. In aqueous solution ammonia assumes equilibrium between ionized (NH_4^+) and unionized (NH_3) chemical species. Its toxicity has been primarily attributed to the un-ionized form, with the ionized form being relatively nontoxic. Different factors can affect the toxicity of unionized ammonia to aquatic organisms. These include pH, dissolved oxygen (DO), salinity and the presence of other toxic substances in water bodies (Russo 1985). The effects of environmental ammonia on fish have been widely studied in the north hemisphere. On the contrary, the knowledge of short-term responses to ammonia in native fish species of South America is still limited. Argentina, as a developing country, has not yet faced severe pollution problems as highly industrialized countries have suffered. However, the faecal and organic pollution in different water bodies of the country are the main problems and accelerate water quality deterioration. The Suquía River Basin, an important lotic system of the central part of Argentina, located in Córdoba Province, has pristine headwaters. On the contrary, the central and lower sections of the basin are characterized by the presence of densely populated urban settlements and an increasing environmental impact due to anthropogenic activities. Pesce and Wunderlin (2000) identified ammonia and nitrite among the main toxicants associated with sewage pollution in the basin. These degraded conditions produce adverse effects on fish, with a marked influence on its abundance and assemblage structure (Bistoni et al. 1999). Reliable data is needed on the sensitivity of native fish species to ammonia and other toxic substances.

The main goal of this work was to determine the toxic impact of ammonia on native fish through laboratory experiments. Field assessments were made in order to validate the bioassay results. This paper reports the median lethal concentration of ammonia (24 hr LC50) to three native fish species: *Bryconamericus iheringi*, *Trichomycterus corduvense* and *Jenynsia multidentata* and compares these results with those obtained from field assessments, which were based on changes in natural populations of the three mentioned species.

Correspondence to: M. A. Bistoni

MATERIALS AND METHODS

Three native fish species with widely neotropical distribution (Malabarba et al. 1998), were chosen due to their geographic ranges and ecological significance: *Bryconamericus iheringi* (Cypriniformes, Characidae), *Trichomycterus corduvense* (Siluriformes, Trichomycteridae) and *Jenynsia multidentata* (Cyprinodontiformes, Anablepidae). They serve as good laboratory models for ecotoxicology studies because they present favorable experimental properties such as small size and being easy to collect and maintain in laboratory conditions. Specimens were collected by a backpack electrofisher (Coffelt, model Mark 10) from an unpolluted site (Suquia River at La Calera, Córdoba, Argentina) (Hued and Bistoni 2005) and transported to the laboratory. Individuals of uniform size (Table 1) were acclimated to controlled conditions (temperature at 25 ± 1°C and light:dark cycle of 12:12 h) during 10 days prior to experiments. Fish were fed once a day with commercial fish pellets and they were not fed for 24 hours prior to the experiment.

Table 1. Mean and standard deviation values of standard length and weight for each fish species used in the experiments.

Species	Standard Length (mm)	Weight (g)
B. iheringi	50.70±9.00	3.60±2.90
T. corduvense	85.20±13.20	8.56±3.45
J. multidentata	35.36±11.30	1.43±1.19

Three replicates and a control group were used for each test concentration. Five individuals of each species were placed in 10 L glass aquaria. The standard guidelines recommend 1g of fish per 1 L of exposure solution. However, the individuals of each fish species were acclimated to control conditions in 10 L glass aquaria, during 10 days prior to the experiments. During this time they did not show neither signs of health deteriorations or mortality. According to these observations we decided to carry out the experiments under the mentioned conditions. Furthermore, control group did not present any mortality during the experiments, which also suggested that five individuals in 10 L tanks did not present any symptoms of stress. The experimental conditions used in our work also responded to economic and spatial limitations.

The dilution water (dechlorinated tap water) was buffered to pH 7.8 by addition of sodium phosphate (Na_2HPO_4). The temperature and photoperiod of the laboratory experiments were the same as from the acclimatation period. Solutions of reagent grade ammonium chloride (NH_4Cl, Merck) were used as the toxicant. Unionized ammonia concentrations (NH_3) were calculated from total-ammonia concentration through the formula of Emerson et al. (1975) and the table of Thurston et al. (1979). For the acute toxicity test, each group was exposed to different ammonia concentrations, that range from 0.62 to 6.50 $mgNH_3{\cdot}L^{-1}$, using a dilution factor of 1.26. Total ammonia nitrogen levels were verified by direct nesslerization (APHA 1998). Median lethal concentration at twenty four-hours (24 hr LC50) was calculated for each fish species through a probit transformation from the mortality dose curve (Landis and Yu 1999). For statistical analysis we used the Infostat software package (2002). One-way analysis of variance and a Tukey test were performed in order to compare 24 hr LC50 values among fish species.

The field sampling collections were carried out in the Suquía River basin, located in a semi-arid region of Córdoba province (Argentina). It begins at the San Roque dam and flows mainly from west to east. This river is the main drinking water source of Cordoba city, located 35 km downstream from the dam, and it also serves for recreation and some sport fishing. Córdoba city, has a population of approximately 1.2 million inhabitants of which nearly 500,000 are connected to the city sewage system; the rest of the sewage goes into groundwater after home treatment (Pesce and Wunderlin 2000). Thereafter, the river flows for about 40 km across the city; downstream near the eastern edge of the city, the river receives the city sewage discharge and then continues up to Mar Chiquita Lake (150 km downstream). We selected two sampling stations on Suquía River Basin, located upstream and downstream from Córdoba City: Saldán (station 1) and Chacra de la Merced (station 2). The sampling sites have been characterized as pristine and high polluted sites, respectively (Bistoni et al. 1999; Pesce and Wunderlin 2000; Hued and Bistoni 2005). They were sampled twice during the rainy season (October-March) and twice during the dry season (April-September), per year, from 1999 to 2002. Fish were captured with backpack electrofisher equipment. Individuals were identified to species level, counted, measured for standard length and weight and then released alive into the stream. The condition factor was calculated using the formula: $CF= (W/SL^3)\cdot 10000$, where W is the weight of each individual and SL correspond to the standard length. For each sampling station we estimated the abundance of each species expressed as numbers of fish captured per unit area of water surface sampled (m^2) (Langford and Hawkins 1997).

The physico-chemical variables monitored were: pH, temperature (°C), conductivity ($mS\cdot cm^{-1}$), alkalinity ($mg\cdot L^{-1}$), dissolved oxygen (DO) ($mg\cdot L^{-1}$), carbon dioxide ($mg\cdot L^{-1}$), total solids ($mg\cdot L^{-1}$), ammonia ($mg\cdot L^{-1}$), nitrites ($mg\cdot L^{-1}$), nitrates ($mg\cdot L^{-1}$), chemical oxygen demand (COD) ($mg\cdot L^{-1}$), 5-day biological oxygen demand (BOD-5) ($mg\cdot L^{-1}$), total phosphorus ($mg\cdot L^{-1}$), hardness ($mg\cdot L^{-1}$), calcium ($mg\cdot L^{-1}$), magnesium ($mg\cdot L^{-1}$), sulfates ($mg\cdot L^{-1}$), chlorides ($mg\cdot L^{-1}$), total iron ($mg\cdot L^{-1}$), total coliforms, ($MPN\cdot 100\ ml^{-1}$: most probable number per 100 ml) and faecal coliforms ($MPN\cdot 100\ ml^{-1}$). Analytical methods were standard (APHA 1998).

One-way analyses of variance was used to find differences in biological variables between sampling stations ($P<0.05$). Physicochemical data were analyzed by a multivariate technique: discriminant analysis through the standard method (Infostat 2002). The location of sampling sites was used as grouping variable: upstream (Station 1) and downstream Córdoba city (Station 2), in order to determine the influence of this urban center on the water quality of Suquía River.

RESULTS AND DISCUSSION

According to our results, there are differences in susceptibility among species (Table 2). The value obtained for *T. corduvense* was significantly higher than the other species ($F= 1226.58$; $p<0.0001$). No significant differences were found between *J. multidentata* and *B. iheringi*, despite the higher mean value obtained for *J. multidentata.*

Several studies have reported 24-LC50 of unionized ammonia that varied from 0.1 to 2.9 $mgNH_3 \cdot L^{-1}$ (Ball 1967; Lloyd and Orr 1969; Rice and Stokes 1975). Besides this, many laboratory experiments of relatively short duration (24-96hs) demonstrated that the acute lethal concentration of ammonia for a variety of non-salmonids fish species lies in the range 0.4 - 4.0 $mgNH_3 \cdot L^{-1}$ (USEPA 1999). According to our results, *B. iheringi* and *J. multidentata* could be considered as moderately tolerant species while *T. corduvense* could be classified as tolerant to high ammonia concentrations.

Table 2. Twenty-four-hour median lethal concentration (24-LC50) of unionized ammonia (NH_3) for three native fish species. Different letters represent significant differences from one way analyses of variance and Tukey test ($p<0.05$).

Species	NH_3 ($mg \cdot L^{-1}$)
Bryconamericus iheringi	1.005 ± 0.0499^{a}
Jenynsia multidentata	1.210 ± 0.082^{a}
Trichomycterus corduvense	4.469 ± 0.1363^{b}

In order to characterize water conditions at each station based on environmental parameters and determine the influence of Córdoba city on water quality, the physico-chemical data were subjected to a discriminant analysis. This method clearly distinguished different water quality conditions before and after the city. Mean values and standard deviations of the most important physico-chemical parameters yielded by the discriminant analyses at each sampling site are shown in Table 3. Samples were grouped in two types: those that belong to Saldán, before the city (Station1) and those from the most polluted site, after the city sewage discharge (Station 2), with a 100% of right assignations using only seven parameters. They were: total phosphorus, ammonia, BOD-5, dissolved oxygen (DO), calcium, total hardness and alkalinity. These physico-chemical variables showed a marked difference due to the negative influence of Córdoba city. The decrease in DO levels and the highest concentrations of total phosphorus, ammonia and BOD-5 suggest that the main degradation cause is organic pollution, which is particularly important after the wastewater treatment plant (Station 2).

Table 3. Mean, minimum (Min.) and maximum (Max) values of the most important physico-chemical parameters yielded by the discriminant analyses.

Physical and chemical parameters ($mg \cdot L^{-1}$)	Station 1		Station 2	
	Mean	Min. - Max.	Mean	Min. - Max.
Total Phosphorus	0.06	0.00-0.31	1.02	0.35-2.89
Ammonia	0.012	0.00-0.07	0.119	0.03-0.29
BOD-5	1.11	0.80-1.55	2.47	1.44-4.48
DO	10.91	9.20-12.40	6.26	3.40-8.80
Calcium	23.29	4.74-40.81	61.78	46.58-96.18
Total Hardness	87.63	19.07-163.77	223.11	167-340.51
Alkalinity	105.14	90.00-140.00	153.75	128.00-195.00

References: Station 1 (before Córdoba city), Station 2 (after the city). BOD-5: Biological Oxygen Demand. DO= dissolved oxygen.

The reference station (Station 1: Saldán) presented low concentrations of ammonia (0.01 $mgNH_3 \cdot L^{-1}$). The most polluted area (Station 2: Chacra de la Merced) registered a significant increase in this parameter (0.12 $mgNH_3 \cdot L^{-1}$) ($F= 8.79$; $p= 0.0109$). The biological variables also showed significant differences between sites. The abundance of *B. iheringi* and *T. corduvense* decreased with increased water quality degradation ($F= 9.61$, $p=0.0084$; $F= 9.96$, $p= 0.0076$, respectively) and *J. multidentata* did not show differences and was abundant in both pristine and polluted sites ($F= 2.49$ $p= 0.1387$).

The condition factor registered for the studied species presented significant differences between sampling areas (Table 4). The lowest values corresponded to the most polluted site (Station 2) indicating an impairment of the general health condition of fish species due to the water quality deterioration.

Table 4. Mean and standard deviation values of standard length, weight and condition factor for each field collected fish species.

		Species		
	Station	*B. iheringi*	*T. corduvense*	*J. multidentata*
Standard	1	51.64±11.0	84.80±14.4	36.13±10.7
Length (mm)	2	38.96±12.6	69.33±21.3	31.48±10.4
Weight (g)	1	3.87±3.02	8.77±4.32	1.39±1.38
	2	1.55±1.58	4.89±4.90	0.83±0.87
Condition	1	0.23±0.06	0.14±0.01	0.22±0.04
Factor	2	0.17±0.05*	0.11±0.01*	0.18±0.05*

References: Asterisks indicate significant differences of condition factor between sampling stations. References: Station 1: Saldán, before Córdoba city and Station 2: Chacra de la Merced, after the city.

The ammonia maximum allowable concentrations for aquatic life indicated by the European Community range between 0.005 to 0.025 $mgNH_3 \cdot L^{-1}$ (CEC 1980). Along Suquía River, Station 1 (Saldán) could be classified as a pristine site while

Station 2 (Chacra de la Merced) had the most degraded water quality, reaching alarming ammonia concentrations (0.119 ± 0.09 $mgNH_3 \cdot L^{-1}$) when the river crosses the city and after the sewage discharge. Bistoni et al. (1999) indicated that increases in ammonia concentrations play a critical role on fish assemblage composition and promotes a decrease of fish abundance in Suquía River. However, comparing the values registered at the field with those obtained in the acute toxicity test for each fish species, it could be noted that the former values are lower than the latter. Values of LC of 5% (*B. iheringi*: 0.662 ± 0.041 $mgNH_3 \cdot L^{-1}$; *J. multidentata*: 0.743 ± 0.094 $mgNH_3 \cdot L^{-1}$; *T. corduvense*: 3.522 ± 0.254 $mgNH_3 \cdot L^{-1}$) are higher than those registered in the field. The different results obtained from laboratory experiments and field assessments suggest that there are some other environmental parameters affecting the native fish abundance in the Suquía River.

It is well known that ammonia toxicity is influenced by DO (Russo and Thurston 1978). Decreases in DO concentrations increase ammonia toxicity, through a ventilation increase by the fish and a corresponding increase in the rate of flow of ammonia across the gill tissues. Besides this, experimental evidence from other studies indicates that a decline in DO content decreases tolerance to ammonia or increases the toxic response to ammonia (Adams et al. 2001). Vámos and Tasnádi (1967) have made some interesting observations on the mortality of carp in fish ponds. Mortality occurred when the concentration of unionized ammonia reached 0.5 $mgNH_3 \cdot L^{-1}$ with a level of 6 $mgO_2 \cdot L^{-1}$ of DO, but when the oxygen concentration was 2 $mgO_2 \cdot L^{-1}$, the lethal level of unionized ammonia was 0.2 $mgNH_3 \cdot L^{-1}$. These authors suggested that these observations are evidence of the effect of low oxygen level on toxicity of ammonia to fish. Dissolved oxygen, a key factor for aquatic organisms, showed high levels upstream Córdoba city (10.91 $mgO_2 \cdot L^{-1}$). Gómez (1993) found that 4 $mgO_2 \cdot L^{-1}$ was the minimum concentration tolerated by fish species. Therefore the mean value registered for station 2 can be considered as sublethal (6.26 $mgO_2 \cdot L^{-1}$) although lower values were registered in some samplings (3.40 $mgO_2 \cdot L^{-1}$, Table 3). The high pollution degree coming from urban activities and waste water treatment plant effluents cause a decrease in DO concentrations, thereby affecting fish species distribution.

The differences found between laboratory and field assessment results indicate that low levels of DO could influence fish species distributions. *J. multidentata,* widely distributed and living in different types of habitats, was considered a tolerant species to salinity and high temperature. Bistoni et al. (1999) noted that this fish species is a common inhabitant of altered areas. In the present study, *J. multidentata* tolerated the degraded water conditions of Chacra de la Merced, because it is restricted to the first centimeters of the water column and shows morphological adaptations to aquatic surface respiration, such as small size and mouth turned upwards (Tagliani et al. 1992). These characteristics give *J. multidentata* the advantage of taking the DO from the surface of the water column, which is saturated with this gas. On the contrary, the two other species do not have this capability. *B. iheringi*, an active swimmer which inhabits the medium part of the water column and *T. corduvense*, a benthic fish species that lives in richly oxygenated waters, could not tolerate the high ammonia concentrations which increased their response to ammonia toxicity due to the low DO levels. As a result, their population levels decreased in response to the water quality deterioration.

The United States Environmental Protection Agency (1999) has published a criterion of 0.02 $mgNH_3 \cdot L^{-1}$ and they have recommended a minimum concentration of 5.5 $mgO_2 \cdot L^{-1}$ to maintain good freshwater fish populations. According to these values, the three fish species considered in our study are under the continuous effect of altered conditions indicated by the high ammonia levels at the most polluted station and accentuated by the low DO concentrations due to the high organic pollution. Although the 24h - LC50 values obtained for each species were higher than the concentrations registered in Suquía River, environmental ammonia exerts a negative influence because its effect is accentuated by the variations of other factors, such as DO.

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