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Copper toxicity for *Scinax ruber* and *Rhinella granulosa* (Amphibia: Anura) of the Amazon: Potential of Biotic Ligand Model to predict toxicity in urban streams

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ABSTRACT

In the last years many populations of anurans have declined and extinctions have been recorded. They were related to environmental pollution, changes of land use and emerging diseases. The main objective of this study was to determine copper sensitivity of the anuran of the Amazon *Rhinella granulosa* and *Scinax ruber* tadpoles at stage 25 and *Scinax ruber* eggs exposed for 96 h to copper concentrations ranging from 15 µg Cu L^{-1} to 94 µg Cu L^{-1}. LC_{50} at 96 h of *Rhinella granulosa* Gosner 25, *Scinax ruber* Gosner 25 and *Scinax ruber* eggs in black water of the Amazon were 23.48, 36.37 and 50.02 µg Cu L^{-1}, respectively. The Biotic Ligand Model was used to predict the LC_{50} values for these species and it can be considered a promising tool for these tropical species and water conditions. Copper toxicity depends on water physical-chemical composition and on the larval stage of the tadpoles. The Gosner stage 19-21 (related to the appearance of external gills) is the most vulnerable and the egg stage is the most resistant. In case of contamination by copper, the natural streams must have special attention, since copper is more bioavailable.

KEYWORDS: Amphibian, LC_{50}-96h, copper, BLM, water pollution.

Toxicidade do cobre em *Scinax ruber* e *Rhinella granulosa* (Amphibia: Anura): Potencial do Modelo do Ligante Biótico para predizer a toxicidade em igarapés urbanos

RESUMO

Nos últimos anos foram registrados muitas extinções e declínios de populações de anuros. Eles estavam relacionados com a poluição do ambiente, a mudanças no uso da terra e ao surgimento de doenças. O principal objetivo deste estudo foi determinar a sensibilidade dos anuros amazônicos ao cobre. Os girinos de *Scinax ruber* e *Rhinella granulosa* no estadio 25 e os ovos de *Scinax ruber* foram expostos por 96 horas a concentrações de cobre entre 15 µg Cu L^{-1} a 94 µg Cu L^{-1}. A CL_{50} -96 h dos girinos de *Rhinella granulosa*, dos girinos de *Scinax ruber* e dos ovos de *Scinax ruber* em águas pretas da Amazônia foram 23,48; 36,37 e 50,02 µg Cu L^{-1}, respectivamente. O modelo do ligante biótico foi usado para prever os valores de CL_{50} para essas duas espécies e pode ser considerado uma ferramenta promissora para essas espécies tropicais e para essas condições de água. A Toxicidade de cobre depende da composição físico-química da água e do estagio larval dos girinos. O estadio 19-21 de Gosner (relacionados ao aparecimento das brânquias externas) são os mais vulnerável e o estagio de ovo é o mais resistente. Em caso de contaminação por cobre, os igarapés naturais devem ter uma atenção especial, uma vez que o cobre é mais biodisponível nesse ambiente.

PALAVRAS-CHAVE: anfíbios, CL_{50}-96h, cobre, BLM, poluição das águas.

INTRODUCTION

Brazil has the greatest diversity of amphibians in the world. Out of the 959 species occurring in Brazil, 650 are endemic to the country. The anuran order is the most threatened (Amphibiaweb 2014). Many populations of anurans have declined and extinctions have been recorded since 1990. They were related to environmental pollution, changes of land use and emerging diseases (Blaustein and Wake 1990; Daszak *et al.* 1999; Houlahan *et al.* 2000). The city of Manaus, State of Amazonas, Brazil, contains a high number of creeks and streams. Black water predominates in these water bodies (Velloso 2002). During periods of heavy rain, these streams overflow forming puddles in their margins, which eventually are used by anurans as a deposition egg sites. The margins of these streams have a vegetation cover that keeps ideal temperature and humidity, or near of that, for reproduction and development of frogs in the urban areas of Manaus.

Black water of the Amazon has very specific physicochemical characteristics and is different from white-water. Typically, black water is found in the Rio Negro that has low pH, low conductivity and low concentrations of cations (Silva *et al.* 1999; Horbe and Oliveira 2008). However, in urban areas under intense anthropic pressure, these characteristics are altered to increased pH, high turbidity, reduced dissolved oxygen concentrations, increased concentrations of calcium, magnesium, sodium, potassium, sulfate and bicarbonate, and high concentrations of transition metals (Silva *et al.* 1999; Melo *et al.* 2005; Nascimento and Silva 2010).

Water quality is central for the Biotic Ligand Model (BLM) that was developed to predict metal toxicity based on water chemistry (Paquin *et al.* 2002). The characteristics of waters of Amazon, both natural and from modified environments, represent a group of interesting conditions to test this model, as it has been reported using fish of the Amazon (Duarte *et al.* 2009). Amphibians have not been tested so far. Frogs are particularly susceptible to the effects of transition metals whose absorption is facilitated by their permeable skin. Tadpoles exposed to high concentrations of metals may present malformations, lower swimming performance and survival rate, longer metamorphosis time and changes in growth (Chen *et al.* 2007; Garcia-Munõz *et al.* 2009; Lance *et al.* 2013).

Scinax ruber and *Rhinella granulosa* are found on the margins of stream crossing the city of Manaus. Many of these streams present high concentrations of copper, often higher than the levels set by the Brazilian National Environmental Council (CONAMA) (< 9 µg Cu L^{-1}) (Silva *et al.* 1999; Santana and Barroncas 2007).The objectives of this study were to determine copper sensitivity of two species of anurans, *Scinax ruber* and *Rhinella granulosa* and to verify the potentiality of the Biotic Ligand Model to predict toxicity in urban streams using tadpoles and eggs.

MATERIALS AND METHODS

Experimental animals

Three spawns from *Scinax ruber* (Laurenti 1768) and three from *Rhinella granulosa* (Spix 1824) were collected next to Barro Branco Stream, Reserva Florestal Adolpho Ducke, Manaus, AM, Brazil (02°55'-03°01'S, 59°53'-59°59'W). The eggs were removed directly from the temporary puddle, transported to the laboratory where they were kept in individual boxes containing continuous aerated water from Barro Branco stream, at room temperature. Tadpoles were fed once a day with pellets of commercial fish food. Feeding was suspended 48 h before starting and during the experiments in order to reduce nonspecific copper binding to organic matter from food and waste products. Tadpoles were acclimated for 21 days in Barro Branco stream water before experiment.

Contaminant

Copper chloride at different concentrations was prepared using water of Barro Branco stream. The water was collected during the rainy season in the Reserva Florestal Adolpho Ducke in Manaus, Amazonas, Brazil and its major characteristics were: [Na^+], 0.182 mg L^{-1}; [K^+], 0.09 mg L^{-1}; [Ca^{2+}], 0.01 mg L^{-1}; [Mg^{2+}], 0.029 mg L^{-1}; [Cl^-], 0.15 mg L^{-1}; [SO_4^{2-}], 1 mg L^{-1}; [DOC] 6.94 mg L^{-1}; [Cu], 1.5 µg L^{-1}; alkalinity 2.8 mg $CaCO_3$ L^{-1}; pH 5.5. These physicochemical characteristics of Barro Branco water are similar to that of black water streams crossing the city of Manaus. To better calibrate the BLM, and LC_{50} was also measured using water from the well of INPA (Brazilian National Institute for Amazon Research). This water is characterized by [Na^+], 0.72 mg L^{-1}; [K^+], 0.42 mg L^{-1}; [Ca^{2+}], 0.1 mg L^{-1}; [Mg^{2+}], 0.026 mg L^{-1}; [Cl^-], 1.32 mg L^{-1}; [SO_4^{2-}], 0.01 mg L^{-1}; [DOC] 1.84 mg L^{-1}; [Cu], 4 µg L^{-1}; alkalinity 3.75 mg $CaCO_3$ L^{-1}; pH 6.2.

Acute effect of contamination (LC50 test)

The LC_{50} is a standard test that determines the concentration of a substance or combination of substances that causes 50% of mortality in a group of organisms, what allow to infer the susceptibility of the species under analysis, *Scinax ruber* and *Rhinella granulosa* in the present study. Four LC_{50} trials were conducted, two for *Scinax ruber* tadpoles (Gosner stage 25, 21 days) in stream water and well water, one with *Scinax ruber* eggs and one with *Rhinella granulosa* tadpoles (Gosner stage 25, 21 days) in water stream, according to the permit of Animal Care Committee of INPA (061/2012). Copper levels were defined according to the protocol of Sprague (1990), i.e., in a geometric increase of 1.4 fold of copper concentration. The total exposure period of tadpoles and eggs to copper chloride was 96 hours. Three repetitions were set for each out of the eight concentrations analyzed. The repetitions consisted of a 500 mL plastic container, filled with 400 mL of continuous aerated water, containing 10 tadpoles or eggs. The same number of repetitions without copper chloride was used as control. Five repetitions were set for *Scinax ruber* tadpoles exposed to well water test. Living larvae and respective developmental stage (according to the table of Gosner 1960) were recorded at 24, 48, 72 and 96 hours. Motionless larvae considered dead and grayish eggs contrasting with live ones were considered not viable.

Chemical analysis

At the beginning of each experiment a sample was taken to analyze the concentrations of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, alkalinity and dissolved organic carbon (DOC). Water pH, temperature and dissolved copper levels were measured daily. If needed, the concentration of dissolved copper was corrected to initial values. The concentrations of Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} were determined by atomic absorption spectrophotometry (Analyst 800, Perkin-Elmer, Singapore), while the concentrations of Cl^{-} were determined by colorimetry (Zall *et al.* 1956), using a plate spectrophotometer (Spectra-Max Plus 384, Molecular Devices, Sunnyvale, CA, USA). All determinations were made in triplicate for each water sample.

The DOC concentration in the samples was determined in a total carbon analyzer (Apollo 9000 TOC analyzer combustion, Teledyne Tekmar, Ohio, USA) using the combustion method combined with infrared detection, as previously described by ISO/FDIS 8245 (1999). In summary, the samples were pre-filtered in 0.45 mm membrane to remove particulate material before injection and the DOC concentrations were determined in triplicates. Concentrations of SO_4^{2-}, S^{-} and humic acid (HA) were averaged from previous determinations for natural streams crossing the city of Manaus (Ertel *et al.* 1986; Silva *et al.* 1999; Nascimento and Silva 2010).

Calculations and Statistical analysis

The LC_{50}-96h and their corresponding 95% confidence intervals were calculated using the Trimmed Spearman-Karber method (Hamilton *et al.* 1977). Average of copper concentrations, measured in triplicate, was used for this calculation.

Measured values of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-} , alkalinity, hardness, pH, DOC, and temperature and values for SO_4^{2-}, S^{-} from the literature were used as input parameters for all experimental series. The content of humic acid was set at 40% in order to correspond to the humic acid content of the Rio Negro (Ertel *et al.* 1986). The BLM software (version 2.2.3 for Windows, Hydroqual, Mahwah, NJ, USA) was used to predict Cu LC_{50}-96h to *Scinax ruber* and *Rhinella granulosa* tadpoles using the *Daphinia magna* (Straus 1820) model and to *Scinax ruber* eggs using rainbow trout model. Each experiment-replicated data was introduced in the program individually.

The physicochemical parameters of the Bolivia stream (Nascimento and Silva 2010) that is located next to the reserve Adolpho Ducke were used as model input parameters. As the author did not measured DOC, S and HA concentrations, we inserted a series of values to verify their influence on the model. For sulfide we consider the same value found for natural streams, because even increasing this value by 150 times, we did not observe changes in LC_{50} predicted by the program. For HA and DOC we insert four different values starting with the concentration of natural stream and rising gradually using a ratio of 2. As these streams have concentrations of organic matter higher than sewage, high DOC and HA values are expected (Table 1).

RESULTS

The *S. ruber* eggs were more resistant to copper than the tadpoles, mainly in the first 24 hours, having a LC_{50} (95% confidence intervals) of 73.93 µg L^{-1} (66.95 – 82.14) that decreased to 51.34 µg L^{-1} (45.58 – 57.82) after 48 hours. At 24 hours of exposure to copper many eggs gave rise to larvae that began to show muscle movements (Gosner stage 18-19) that evolved to larvae presenting gills and swim movements (Gosner stage 25), except those exposed to 57 µg L^{-1}, that remained at Gosner stage 19. At 72 hours all living larvae reached Gosner stage 25.

Tadpoles exposed to stream water of the Ducke Reserve presented decreased survival rate at low copper concentrations even for relatively short period of time. In just 24 hours no *Rhinella granulosa* tadpoles managed to survive when exposed to 33 µg Cu L^{-1}. In contrast, near three times more copper (94

Table 1. Physicochemical characteristics and predicted LC_{50} for Bolivia stream (Manaus city, urban area) and Barro Branco stream (pristine area).

	Temp °C	pH	LC50 µg L^{-1}	DOC mg C L^{-1}	HA %	Ca mg L^{-1}	Mg mg L^{-1}	Na mg L^{-1}	K mg L^{-1}	SO_4 mg L^{-1}	Cl mg L^{-1}	Alcalinity Mg CaCO3 L^{-1}	S mg L^{-1}
Stream in natural area	28	5.5	30.25	6.94	40	0.01	0.029	0.182	0.09	1	0.15	2.8	0.01
Stream in urban area	28	6.5	101.2 – 1498.8	6.9 - 55.3	20 - 80	2.22	52.4	11.55	2.54	5.67	7.65	4.04	0.01

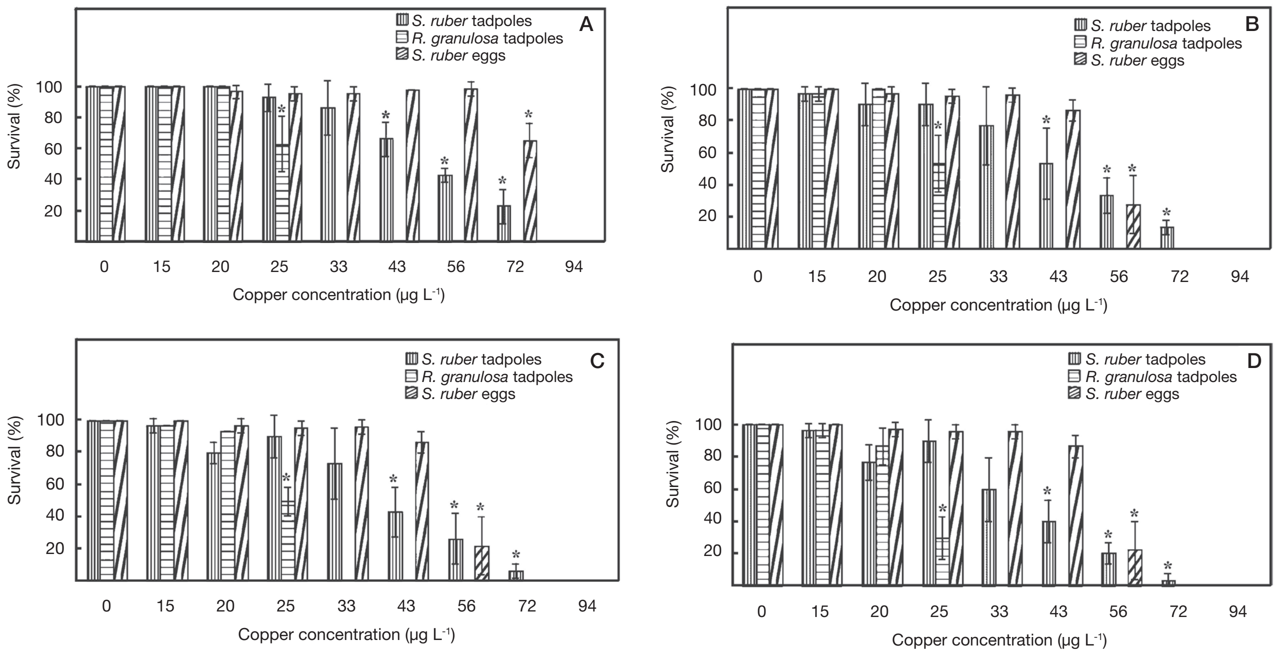

Figure 1. Tadpoles survival rates at different concentrations of copper (15-94 μg Cu L^{-1}) diluted in Barro Branco stream water (mean ± standard error of the mean). (A) 24 hours, (B) 48 hours, (C) 72 hours and (D) 96 hours of exposure. * $P < 0.05$.

µg Cu L^{-1}) were needed to cause total mortality of eggs and tadpoles of *Scinax ruber*. There was no mortality for either species when the tadpoles were exposed to concentrations bellow than 15 µg Cu L^{-1} (Figure 1).

The physical and chemical conditions of the city streams caused by urban pollutions affect copper bioavailability and so its toxicity to tadpoles, relative to animals exposed to pristine water from Barro Branco stream of Ducke Reserve or well water from INPA (Table 1).

The LC_{50}-96h (95% confidence intervals) was 36.37 µg L^{-1} (31.45 – 42.07) for tadpoles and 50.02 µg L^{-1} (44.46 – 56.15) for eggs of *Scinax ruber* and 23.48 µg L^{-1} (21.28 – 25.91) for tadpoles of *Rhinella granulosa* exposed to copper diluted in stream water from Ducke reserve. For *Scinax ruber* tadpoles exposed to copper diluted in the water from INPA well, the LC_{50}-96h was 15.9 µg L^{-1} (13.4 – 19.12).

The predicted LC_{50}-96h was 25.7 µg L^{-1} for tadpoles and 90.9 µg L^{-1} for eggs of *Scinax ruber* and 23.4 µg L^{-1} for tadpoles of *Rhinella granulosa* exposed to copper diluted in stream water from Ducke reserve. For *Scinax ruber* tadpoles exposed to copper diluted in the water from INPA well, the predicted LC_{50}-96h was 19.15 µg L^{-1}. The BLM predicted LC_{50}-96h values are in the same range of measured values indicating that BLM is a reliable tool to estimate copper sensitivities even for polluted tropical urban streams (Figure 2).

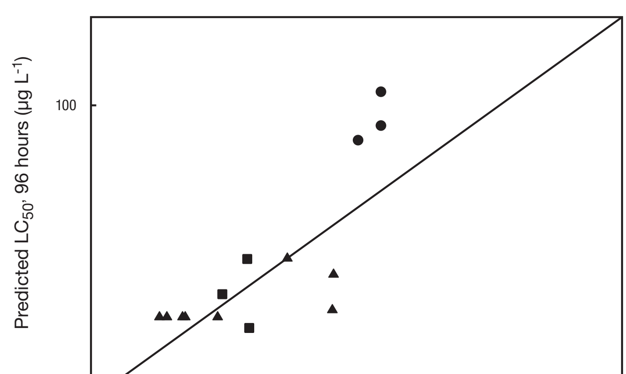

Figure 2. BLM-predicted vs. observed Cu LC_{50} for (●) *Scinax ruber* eggs, (■) *Rhinella granulosa* tadpoles and (▲) *Scinax ruber* tadpoles. The solid diagonal line corresponds to 1:1 agreement between model and data.

DISCUSSION

After 48 hours under exposure to copper there was a high decrease of egg viability, contrasting with the gradual decline of tadpoles mortality. Egg jelly coat may have protected the eggs from contamination in the first 24 hours of exposure. However, the development to Gosner stage 19 - 21 between 24 and 48 hours, that included the appearance of external gills with filaments and cardiopulmonary activities (Gosner 1960), may have increased the absorption of copper and thus a reduction of survival (Figure 1). Lance *et al.* (2012) observed a positive correlation between eggs density and copper toxicity, i.e., the higher the eggs density the lower the copper toxicity. They suggested that eggs jelly binds copper, making it biologically unavailable thereby reducing its toxicity. This seems to be the case also in the present study.

Eggs of *Osteocephalus taurinus* (Steindachner 1862) exposed to contaminated stream waters of the city of Manaus, that contains high background levels of Fe, Ni, Mn, Cr and Pb were more resistant to copper than tadpoles in the first 13 hours of exposure (Franco de Sá *et al.* 2012). Franco de Sá et al. (2012) found no decrease of eggs viability but just after hatching a high mortality was observed, being related to the appearance of external gills, also noted in the present work.

Similar experiment with *O. taurinus* in two other streams of Manaus, Quarenta and Mindu streams, were conducted by Nascimento *et al.* (2012) and unlike Franco de Sá *et al.* (2012) found no effect of polluted water on eggs and larvae during the period of 96 hours. This difference between the two studies seems to be related to physical and chemical composition of the streams that affect the bioavailability of metals, thus requiring specific contrasting analysis of urban polluted sites.

The LC_{50}-96h was higher for *Scinax ruber* than *Rhinella granulosa* possibly due to a higher resistance of *Scinax ruber* to copper and to slight variations of physicochemical characteristics of waters used in the experiments. The waters were collected in the same stream, but on different days for each experiment. Short-term (48h) exposure to copper caused a decreased larval development. A delay in tadpoles development can have an negative effect on the population, because the longer they stay in the puddles the greater the chances of being preyed and the puddles to dry.

In contaminated streams we observed a reduction in toxicity, with predicted LC_{50} values ranging between 101.2 µg L^{-1} and 1,498 µg L^{-1}, depending on the concentrations of DOC and HA (Table 1). Specific physicochemical conditions of the polluted streams, including increased DOC, affect bioreactivity and bioavailability of metals and may make these metals biologically unavailable to the body, thereby reducing their toxicity. The toxicity of metals is due to metal binding to the biotic ligand, and gills are the main ligand. The pH has a significant influence on metal speciation, i.e., increased pH as observed in the polluted streams facilitates the formation of metal-COD complexes thus reducing metal binding to biotic ligand. SO_4^{2-} and Cl^- cause precipitation of metals, decreasing also their bioavailability. Humic substances form highly stable complexes with high molecular weight, thus reducing their uptake by the biological membranes (Paquin *et al.* 2002).

Metal bioavailability in natural streams of the Amazon depends on multiple and complex chemical and physical interactions, such as pH ranging from very acidic to near neutral conditions, from very low to relatively high levels of ions, from low to high DOC levels, and increased organic and inorganic pollutants as occur in urban streams. These conditions determine metal toxicity and so cause damage in different extensions to the populations of frogs that inhabit water bodies' banks.

Field assays performed in different types of water from the Amazon including white, black and a mixture of white and black waters, have suggested that the BLM is a suitable tool to predict copper toxicity (Bevilacqua 2009) for fish in these natural waters. Bianchini *et al.* (2004) also showed that BLM is a promising tool to estimate copper sensitivity for copepods in subtropical waters. The present study highlights that Biotic Ligand Model can also be considered a promising tool to predict copper sensitivity using anurans of the Amazon.

Amphibians are daily exposed to a large number of urban threats within cities or near them. Expansion of cities near forest edges causing deforestation have direct effects on most of anuran species. Among the causes for this constraints are increased background levels of metals, including copper on water and soil. Indeed, because amphibians, both larvae and adults, have permeable skins, there is a metal accumulation in different tissues (Loumbourdis and Wray 1998). However, there are a few species that can to survive these changes, as *Scinax ruber* and *Rhinella granulosa*, which have a preference for open areas and do not require vegetation to spawn (Lima *et al.* 2006).

Anurans found in urban polluted areas are able to continuously adapt to environmental changes. Green frogs exposed to the influence of urbanization and pollution manifested shifts in the balance of immunocompetent cell subpopulations and changes in white blood count (WBC), reflecting the responses of amphibians to heavy pollution of their environment (Romanova and Egorikhina 2006). Other amphibian species, as the populations of ***Pelophylax*** *ridibundus* inhabiting polluted ponds, present changes hematological parameters reflecting their adaptive responses to the environmental factors as reported by (Zhelev *et al.* 2013). In general, these variations reflect physiological reactions to the presence of toxicants in the environment and together with physicochemical characteristics of water can be used as environmental indicators of pollution of water basins (Romanova and Egorikhina 2006; Zhelev *et al.* 2012; Zhelev *et al.* 2013). Increasingly amphibians and their biological characteristics have been used as sentinel organisms for environmental changes.

Copper concentrations of 3,798 mg g^{-1} in sediment and 158 mg L^{-1} in water were reported for urban streams of Manaus city (Silva *et al.* 1999; Dias 2001). Depending on physical characteristics and chemical composition of the water, these copper concentrations can be lethal to *S. ruber* e *R. granulosa* tadpoles. Long term sub-lethal concentrations of copper can cause adverse effects in the anurans development, such as reduction of weight and final length of tadpoles, increasing the chances of being preyed or appearance of malformations, such as wavy dorsal fin, bend tail, curvature body axis, yolk sac edema and reduced pigmentation, DNA damage and cell apoptosis (Lance *et al.* 2013; Xia *et al.* 2012). All these factors can, in the long term, result in reduction of local populations of frogs.

About 70% of amphibians depend on water to spawn (Sparling *et al.* 2000). Most of these species does not use the streams directly but they use puddles often formed on the banks of the streams by rainfall or by streams overflowing. Urban puddles are continuous changing environments because they continuously receive polluted water from urban streams; undergo an intense bacterial decomposition of organic matter; and experience extreme temperatures and UV incidence, thus stressing the effects of pollutants. Despite that, using BLM was possible to reasonably estimate copper sensitivity in these urban aquatic bodies.

CONCLUSIONS

Copper toxicity depends on water physical-chemical composition and on the larval stage of the tadpoles. The Gosner stage 19-21 (related to the appearance of external gills) is the most vulnerable and the egg stage is the most resistant. In case of contamination by copper, the natural streams must have special attention, since copper is more bioavailable. The findings presented here represent the first attempt to understand copper toxicity to Amazonian anurans. Our results highlight the first evidence that Biotic Ligand Model can be considered a promising tool for the prediction of copper toxicity for regulatory purposes in the waters from Amazonian urban streams.

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REFERENCES

Amphibiaweb, 2014. Information on Amphibian Biology and Conservation. Berkeley (http://amphibiaweb.org). Accessed on 25/03/2014.

Bevilacqua, A.H.V. 2009. *O uso do modelo do ligante biótico para a avaliação da contaminação por cobre em águas da Amazônia*. Dissertação de Mestrado, Instituto Nacional de Pesquisas da Amazônia/ Fundação Universidade do Amazonas, Manaus, Amazonas. 36p.

Bianchini, A.; Martins, S.E.G.; Barcarolli, I. F. 2004. Mechanism of acute copper toxicity in euryhaline crustaceans: implications for the Biotic Ligand Model. *International Congress Series*, 1275: 189–194.

Blaustein, A.R.; Wake, D.B. 1990. Declining amphibian populations: a global phenomenon? *Trends in Ecology and Evolution,* 5: 203-204.

Chen, T.H.; Gross, J.A.; Karasov, W.H. 2007. Adverse effects of chronic copper in larval northern leopard frogs (*Rana pipens*). *Environmental toxicology and Chemistry,* 26: 1470–1475.

Daszak, P.; Berger, L.; Cunningham, A.A.; Hyatt, A.D.; Green, D.E.; Speare, R. 1999. Emerging Infectious Diseases and Amphibian Population Declines. *Emerging Infectious Diseases,* 5: 735-748.

Dias, C.M. 2001. *Estudo físico-químico da água de três igarapés da regiâo do distrito industrial de Manaus*. Dissertação de Mestrado, Fundação Universidade do Amazonas, Manaus, Amazonas. 114p.

Duarte, R.M.; Menezes, A.C.L.; Rodrigues, L.; Almeida-Val, V.M.F.; Val, A.L. 2009. Copper sensitivity of wild ornamental fish of the Amazon. *Ecotoxicology and Environmental Safety*, 72: 693-698.

Ertel, J.; Hedges, J.; Devol, A.; Richey, J.; Ribeiro, M. 1986. DissoIved humic substances in the Amazon river system. *Limnology Oceanography*, 31: 739-754.

Franco de Sá, J.F.O.; Keller, C.; Santana, G.P.; Menin, M. 2012. Efeito da poluição do igarapé do Educandos (Manaus, Amazonas, Brasil) sobre ovos e girinos de *Osteocephalus taurinus* (Anura, Hylidae). In: Marcon, J.L.; Menin, M.; Araújo, M.G.P.; Hrbeck, T. (Ed.). *Biodiversidade amazônica: caracterização, ecologia e conservação.* EDUA, Manaus, Amazonas, p.301-323.

García-Muñoz, E.; Guerrero, F.; Parra, G. 2009. Effect of copper sulfate on growth, development, and escape behavior in *Epidalea calamita* embryos and larvae. *Archives of Environmental Contamination and Toxicology,* 56: 557–565.

Gosner, K. L. A. 1960. Simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, 16: 183-190.

Hamilton, M.A.; Russo, R.C.; Thurston, R.V. 1977. Trimmed Spearman-Karber method for estimating lethal concentrations in toxicity bioassays. *Environmental Science & Technology*, 11: 714-719.

Horbe, A.M.C.; Oliveira, L.G.S. 2008. Química de igarapés de água preta do nordeste do Amazonas – Brasil. *Acta Amazonica*, 38: 753–760.

Houlahan, J.E.; Findlay, C.S.; Schmidt, B.R.; Meyer, A.H.; Kuzmin, S.L. 2000. Quantitative evidence for global amphibian population declines. *Nature,* 404: 752-758.

ISO/FDIS 8245. 1999. *(Standard 8245) water quality – Guidelines for the determination of total organic carbon (TOC) and dissolved organic carbon (DOC)*. International Organization for Standardization, Geneva, Switzerland. 11p.

Lance, S.L.; Erickson, M.R.; Flynn, R.W.; Mills, G.L. 2012. Effects of chronic copper exposure on development and survival in the southern leopard frog (*Lithobates [Rana] sphenocephalus*). *Environmental Toxicology and Chemistry*, 31: 1587-1594.

Lance, S.L.; Flynn, R.W.; Erickson, M.R.; Scott, D.E. 2013. Within and among population level differences in response to chronic copper exposure in southern toads, *Anaxyrus terrestris. Environmental Pollution*, 177: 135-142

Lima, A.P.; Magnusson, W.E.; Menin, M; Erdtmann, L.K.; Rodrigues, D.J.; Keller, C.; Hold, W. 2006. *Guia de sapos da Reserva Adolpho Ducke, Amazônia Central*. Áttema Design Editoria, Manaus, 168p.

Loumbourdis, N.S.; Wray, D. 1998. Heavy metal concentration in the frog *Rana ridibunda* from a small river of Macedonia, Northern Greece. *Environment. International*, 24: 427-431.

Melo, E.G.F.; Silva, M.S.R.; Miranda, S.A.F. 2005. Influência antrópica sobre águas de igarapés na cidade de Manaus-Amazonas. *Caminhos de Geografia*, 5: 40–47.

Nascimento, C.R.; Silva, M.R.S. 2010. Alterações em parâmetros físicos e em concentrações de cátions e ânions em uma micro-bacia hidrográfica de Manaus devido a expansão urbana. *Caminhos de Geografia,* 11: 208–219.

Nascimento, J.H.P.; Keller, C.; Silva, M.S.R.; Menin, M. 2012. Toxicidade crônica da água contaminada dos igarapés Mindu e Quarenta (Manaus, Amazonas) sobre ovos e girinos de *Osteocephalus taurinus* (Anura, Hylidae). In: Marcon, J.L.; Menin, M.; Araújo, M.G.P.; Hrbeck, T. (Ed.). *Biodiversidade amazônica: caracterização, ecologia e conservação.* EDUA, Manaus, Amazonas, p.325-346.

Paquin, P.R.; Gorsuch, J.W.; Apte, S.; Batley, G.E, Bowles, K.C.; Campbell, P.G.C.; *et al*. 2002. The Biotic Ligand Model: a historical overview. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 133: 3-35.

Romanova E.B.; Egorikhina M.N. 2006. Changes in hematological parameters of *Rana* frogs in a transformed urban environment. *Russian Journal of Ecology*, 37: 188-192.

Santana, G.P.; Barroncas, P.S.R. 2007. Estudo de metais pesados (Co, Cu, Fe, Cr, Ni, Mn, Pb e Zn) na Bacia do Tarumã-Açu Manaus – (AM). *Acta Amazonica*, 37: 111-118.

Silva, M.S.R.; Ramos, J.F.; Pinto, A.G.N. 1999. Metais de transição nos sedimentos de Igarapés de Manaus-AM. *Acta limnologica Brasiliensia,* 11: 89-100.

Sparling, D.W.; Bishop, C.A.; Linder, G. 2000. The current status of amphibian and reptile ecotoxicological research. In: Sparling, D.W.; Linder, G.; Bishop, C.A. (Ed.). *Ecotoxicology of Amphibians and Reptiles*. Society of Environmental Toxicology. (SETAC) Pensacola, FL, p.1-13.

Sprague, J.B. 1990. Aquatic toxicology. In: Schrech, C.B.; Moyle, P.B. (Ed.). *Methods of Fish Biology*. American Fisheries Society Bethesda, Maryland, p. 491-528.

Velloso, R. 2002. *Projeto geo cidades: relatório ambiental urbano integrado informe GEO*: Manaus/ Supervisão: Ana Lúcia Nadalutti La Rovere, Samyra Crespo. Consórcio Parceria 21, Rio de Janeiro, 188p.

Zall, D.M.; Fisher, D.; Garner, M.D. 1956. Photometric determination of chlorides in water. *Analytical Chemistry*, 28: 1665-1678.

Zhelev, Zh.M.; Arnaudov, A.D.; Popgeorgiev, G.S.; Dimitrov, H.A. 2012. Assessment of ecological status of two rivers with different types of anthropogenic pollution in Southern Bulgaria based on the level of fluctuating asymmetry in the populations of marsh frog *Rana ridibunda* (Amphibia: Ranidae). *Acta Zoologica Bulgarica (Supplement),* 4: 225-231.

Zhelev, Zh.M.; Popgeorgiev, G.S.; Angelov, M.V. 2013. Investigating the changes in the morphological content of the blood of *Pelophylax ridibundus* (Amphibia: Ranidae) as a result of anthropogenic pollution and its use as an environmental bioindicator. *Acta Zoologica Bulgarica*, 65: 187-196.

Xia, K.; Zhao, H.; Wu, M.; Wang, H. 2012. Chronic toxicity of copper on embryo development in Chinese toad, *Bufo gargarizans. Chemosphere,* 87: 1395-1402.

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