Ciências Exatas

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Avaliação da Concentração Letal (CL_{50}) de substâncias húmicas aquáticas do rio Negro (Brasil) e de seu potencial na redução de toxicidade aguda de íons Cu^{2+}[(1)]

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Resumo

Esta investigação avaliou o potencial das substâncias húmicas aquáticas (SHA) na redução da toxicidade dos íons cobre (Cu^{2+}) utilizando a *Artemia salina* como organismo teste. Primeiramente, determinou-se a concentração letal (CL_{50}; concentração onde há 50% de mortalidade da população de um organismo teste) de íons Cu^{2+} e SHA para Artemias. Em seguida, diferentes quantidades de SHA foram misturadas com íons Cu^{2+} na CL_{50} a fim de estimar a taxa de mortalidade de *Artemia salina* em comparação com os testes realizados sem SHA. Também testou-se as frações SHA, ácidos húmicos (AH) e ácidos fúlvicos (AF), em diferentes proporções. Os resultados mostraram que a CL_{50} de Cu^{2+} foi de 19 mg L^{-1} e entre 400 e 600 mg L^{-1} para SHA. Os ensaios com SHA mostraram uma redução de até 40% na mortalidade das *Artemia salina*, ao passo que os ensaios com AH e AF diminuíram essa taxa em até 20%, o que indica que SHA tem maior poder redutor de toxicidade que suas frações de ácido húmico e fúlvico separadas. Os resultados desses experimentos com diferentes proporções de AH e AF sugerem que a redução da toxicidade de Cu^{2+} é diretamente proporcional ao aumento da proporção de AH.

Palavras–chave: *Artemia salina*, toxicologia ambiental, redução de toxicidade, ácido fúlvico e húmico

Assessment of the Lethal Concentration (LC_{50}) of aquatic humic substances from Rio Negro (Brazil) and their potential to reduce the toxicity of Cu^{2+} ions. This investigation assessed the potential of aquatic humic substances (AHS) to decrease the toxicity of copper (Cu^{2+}) ions using *Artemia salina* as test organism. Firstly, the LC_{50} (lethal concentration that destroys 50% of a test organism population) values of Cu^{2+} and AHS for the test organism were determined. Subsequently, assays with solutions of Cu^{2+} at LC_{50} were carried out in the presence of AHS at different concentrations in order to estimate the mortality rate of *Artemia salina* in comparison to the mortality rate obtained when AHS is

[1] Os resultados apresentados nesse artigo foram obtidos na tese de doutorado de Silva (2019) intitulada "Substâncias húmicas aquáticas do rio Negro-AM: impacto da variação da sua composição na labilidade de íons metálicos e em seu efeito redutor de toxicidade aguda"

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absent. The AHS fractions, humic acids (HA) and fulvic acids (FA), were also tested at different ratios. The results showed that the LC_{50} was 19 mg L^{-1} for Cu^{2+} and between 400 and 600 mg L^{-1} for AHS. The trials with AHS showed a reduction of up to 40% in the mortality rate of *Artemia salina* whereas HA and FA decreased the rate by up to 20%. These findings suggest that AHS reduce the toxicity of Cu^{2+} to a greater extent than their fractions HA and FA individually. The results of experiments with different ratios of HA and FA indicated that there is greater reduction of the toxicity of Cu^{2+} when the ratio of HA is higher.

Keywords: *Artemia salina*, environmental toxicology, toxicity reduction, fulvic and humic acids

1. Introduction

Aquatic humic substances (AHS) are compounds derived from the decomposition of plants and animals and comprise most of the dissolved organic carbon in natural waters (McDonald *et al*., 2004). They significantly affect the behaviour of metal ions and other organic and inorganic species in aquatic environments. (Klučáková *et al.*, 2018; Cuprys *et al.*, 2018). It is well known that AHS can decrease the toxicity of several metal ions (Watanabe *et al.*, 2017) due to their strong affinity. However, little is known about how this interaction affects biological parameters such as the toxicity levels to the aquatic biota. Moreover, very few studies have investigated how changes in the concentration of AHS and their major fractions, humic acids (HA) and fulvic acids (FA), reduce the toxicity levels of potentially toxic metals (Li *et al.*, 2018; Zhang *et al*., 2019).

The transport and fate of metals are affected by different factors such as precipitation/dissolution, oxidation/reduction, adsorption/desorption, and complexation (Stumm and Morgan, 1995). Therefore, all these processes must be considered in order to understand the behaviour of potentially toxic metals in natural systems, which requires the characterisation of their different chemical species, including AHS-metal complexes. The environmental quality standards in the United States, for instance, are currently based on the concentration of the dissolved ionic fraction of metal species (EPA, 2018). However, it is well known that the toxicity and bioavailability of metals also depend on complexing or chelating agents in the solution (Väänänen *et al.*, 2018).

The presence of AHS can change biological adsorption processes of a significant number of chemical species, since complexes cannot easily permeate through the cell membranes, decreasing the bioavailability of metals and therefore their toxicity (Hue, Craddock, and Adams 1986; Ding *et al*., 2018). Bai (2019), for instance, observed that HA decreases the availability of Pb^{2+} ions to algae.

The measurement of toxicity of chemicals can be assessed by observing the mortality rate of this chemical to a test organism. A useful parameter to determine toxicity is the lethal concentration (LC_{50}) (Stephan, 1977). The LC_{50} is the concentration of a chemical that destroys 50% of a population of a test organism (Boyd, 2005).

Artemia salina is a test organism generally used to determine the LC_{50} of chemicals, especially in the pharmaceutical industry (Arcanjo *et al*., 2012; Cavalcante *et al*., 2000; Pimentel *et al*., 2011). *Artemia salina* (or brine shrimp, its popular name) is a small crustacean of the order Anostraca and a unique genus of the *Artemidae* family. These organisms are highly resistant to adverse conditions and can live at high levels of water salinity

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(Dumitrascu, 2011). *Artemia salina* is often used in ecotoxicological assays as it can be easily purchased in the form of cysts at a low cost. In addition, this organism present rapid hatching time and ecotoxicity testing protocols (both 24h) utilizing it have been found to produce fast results (Rajabi *et al*., 2015).

The main purpose of this study is to assess the potential of AHS and the fractions HA and FA to decrease the toxicity of Cu^{2+} in the presence of *Artemia salina*, the test organism chosen. A series of acute toxicity assays were performed using solutions of Cu^{2} at lethal concentration (LC_{50}) with different concentrations of AHS and with different ratios of HA and FA (since the amount of HA and FA can be associated to humus formation). These tests sought to understand how the concentration and composition of these substances affect the reduction of acute toxicity.

2. Materials and methods

Hatching of *Artemia salina* cysts

Dormant cysts of *Artemia salina*, purchased from an aquarium shop, were placed in a cleaned container. The cysts (200 mg) were incubated in 200 mL syntenic seawater (4 g L^{-1} of salt) under constant illumination (15W fluorescent lamp, 6400K) and continuous aeration, maintained by an air pump (Boyu U-02800) for 24h (Figure 1) (Silva *et al.*, 2010; Vanhaecke *et al*., 1981). After the incubation period, the hatched *Artemia salina* nauplii were separated from the non-hatched cysts.

Isolation of aquatic humic substances and the fractions humic acid and fulvic acid

Surface water (280 L) for AHS isolation was taken from the Rio Negro River, at the confluence of the catchments of Tarumã-Açu and Tarumã-Mirim, 20 km from the northern Brazilian city of Manaus (Amazonas) (3"04'13.74" S and 60"08'26.92" W), in December 2005.

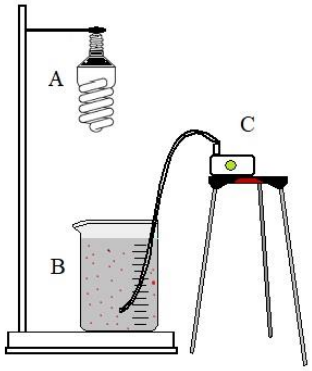

Figure 1. Schematic representation of the apparatus used for hatching of *Artemia salina* cysts. A) 15W fluorescent lamp, 6400 K; B) Beaker (200 mL); C) air compressor pump. Source: the authors.

The procedure performed to extract AHS was based on the International Humic Substances Society's (2018) guidelines: using acrylic ester resin XAD-8 to adsorb the AHS in acidic medium (river water is acidified until pH<2 using hydrochloric acid (HCl)) before they are desorbed in alkaline conditions (Aiken *et al.*, 1985) (sodium hydroxide, NaOH 0.1 mol L^{-1}). Afterwards, the extract was desalted using dialysis membranes (Samless Cell 16 x 100 clear) prepared according to Town and Powell (1992). The dialysis was performed with dialysis tubing against deionised water until the dialysate produced a negative chloride test with silver nitrate ($AgNO_3$).

Half of the dialysed extract was reserved for the fractionation of HA and FA. The other half was lyophilised and stored in amber tubes until time of use. The fractionation of AHS was carried out via acidification of the extracts until pH 2, using HCl. Then, the precipitate (HA) was separated from the supernatant (FA), and both fractions were neutralised prior to

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being lyophilised (Thurman and Malcolm, 1981).

Preparation of solutions

A synthetic seawater solution (4 g L^{-1}) was prepared using sea salt (Red Sea Salt)—composed of magnesium (Mg, 1230 ppm), potassium (K, 403 ppm), and sulphur (S, 889 ppm) and whose alkalinity was 2.8 meq L^{-1}. A copper nitrate ($Cu(NO_3)_2$) solution at concentration 0.57 g L^{-1} was prepared with the synthetic seawater in order to be diluted to the target concentrations (5 to 50 mg L^{-1}) in the acute toxicity assays.

The extracted AHS powder was diluted in the synthetic seawater to 600 mg L^{-1} AHS, following a pH adjustment to 5 (pH of Rio Negro River). AHS was then diluted in several concentrations (10, 50, 100, 200, 400 and 600 mg L^{-1}) for the toxicity assays.

Acute toxicity assays

Three sets of experiments (described in the next topics) were performed with the hatched nauplii of *Artemia salina*, in which Pasteur pipettes, 24-well culture plates of 3 mL, and a magnifying glass (for counting the surviving larvae) were used. In all tests, ten larvae were transferred into each cell of the 24-well cell culture plates, to which 1 mL of the 4 g L^{-1} sea salt solution was added. In subsequent tests, an aliquot (depending on the target concentration) of the 0.57 g L^{-1} $Cu(NO_3)_2$ solution was used to investigate both the acute toxicity of Cu^{2+} and the influence of AHS on the results. In some tests, a pre-determined mass of AHS was added to each cell, which had their volumes completed with the sea salt solution. All cell culture plates were incubated at 25°C for 24 h. At the end of the incubation period, the surviving larvae were counted using a magnifying glass. The culture plates were gently shaken, and the larvae were considered dead if they did not exhibit movement during 10s of observation. The nauplius were not fed during the tests as they consume the energy reserves stored in the cysts in the first 24h (Sorgeloos, 1978; Briski *et al.*, 2008).

The mortality rate (m) of *Artemia salina* was calculated using 48 cells of two culture plates (24 cells each) and according to the following equation: $m(\%) = (A_M/P_T) \times 100$, where A_M is the number of dead *Artemia salina* and P_T is the total number of *Artemia salina* used in each cell of the 24-well cell culture plates (Moreira, 2013).

Acute toxicity of Cu^{2+} ions to *Artemia salina*

The first set of experiments used copper nitrate ($Cu(NO_3)_2$) at concentration between 5 and 50 mg L^{-1} in order to determine the lethal concentration (LC_{50}) of Cu^{2+} ions to *Artemia salina (*According to Vanhaecke (1981), LC_{50} is defined as the concentration of solvent/substance that destroys 50% of a group of the test organisms). The LC_{50} of Cu^{2+} ions were used in the experiments to investigate the role of AHS and their fractions in reducing the toxicity of Cu^{2+}.

Acute toxicity of AHS to *Artemia salina*

The second set of experiments was performed using different concentrations of AHS (10, 50, 100, 200, 400, and 600 mg L^{-1}) to determine the LC_{50} of AHS to *Artemia salina*. The LC_{50} of AHS was the maximum AHS concentration that could be used in the toxicity assays without causing acute effects in the organisms.

Assessment of AHS's potential to decrease the acute toxicity of Cu^{2+} ions to *Artemia salina*

The potential of AHS and their fractions HA and FA to reduce the acute toxicity of Cu^{2+} ions to *Artemia salina* was

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analyzed in the third set of experiments. The tests used two different Cu^{2+} ion concentrations (19 mg L^{-1}, corresponding to the calculated LC_{50}, and 30 mg L^{-1}, corresponding to a mortality rate of 85% of the organisms) and four different AHS concentrations (25, 50, 75, and 100 mg L^{-1}).

Assessment of the potential of different concentrations and ratios of HA and FA to decrease the acute toxicity of Cu^{2+} ions to *Artemia salina*

Toxicity tests using different ratios (w/w) of HA and FA fractionated from the AHS were performed in order to understand the contribution of these major components to reduce the acute toxicity of Cu^{2+} to *Artemia salina*. Then, different ratios of HA and FA (0 % HA and 100 % FA, 25 % HA and 75 % FA, 50 % HA and 50 % FA, 75 % HA and 25 % FA and 100 % HA and 0 % FA) were tested at the following concentrations (typically recorded for rivers): AHS: 25 mg L^{-1}, 50 mg L^{-1}, 75 mg L^{-1}, and 100 mg L^{-1}. The Cu^{2+} concentration used in the assays was always 19 mg L^{-1} (corresponding to the LC_{50} of Cu^{2+}).

Statistical analysis

The Lilliefors test was used to examine if the data were normally distributed for a level of significance of 0.05. After confirming the normality of data, the Fisher's Least Significant Difference (LSD) test was performed at a significance level of 0.05.

3. Results

LC_{50} of Cu^{2+} to *Artemia salina*

The results of the mortality rate for *Artemia salina* after being exposed for 24 hours to solutions of copper ion at concentration ranging from 0 to 50 mg L^{-1} are summarised in Table 1. Since the concentration was lethal for more than 50% of the testing organisms from 19 mg L^{-1} onwards, this was the LC_{50} determined for Cu^{2+}.

Table 1. Mortality rate of *Artemia salina* exposed to copper ions.

Concentration of Cu^{2+} (mg L^{-1})	Mortality rate (%)	Concentration of Cu^{2+} (mg L^{-1})	Mortality rate (%)
5	1.3	25	88.1
10	18.8	30	82.5
15	30.6	35	82.5
18	46.9	40	88.8
19	56.7	45	86.3
20	68.8	50	98.8

LC_{50} of AHS to *Artemia salina*

The results of the mortality rate for *Artemia salina* after being exposed to different AHS concentrations are shown in Table 2. From these assays, the LC_{50} of AHS to *Artemia salina* could not be determined precisely.

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Table 2. Mortality rate of *Artemia salina* exposed to different aquatic humic substances (AHS) concentrations

Concentration of AHS (mg L^{-1})	Mortality rate (%) (n=48)	Standard deviation
10	0.0	0.0
50	0.0	0.0
100	0.0	0.0
200	0.0	0.0
400	0.0	0.0
600	100.0	0.0

Reduction of acute toxicity of Cu^{2+} to *Artemia salina* in the presence AHS

Table 3 shows the results obtained from the toxicity assays using Cu^{2+} ions at LC_{50} (19 mg L^{-1}) in the presence of AHS concentrations ranging from 25 to 100 mg L^{-1}. Tests with the lowest AHS concentration presented mortality rates two times higher than the tests using higher amounts of AHS. Nonetheless, reduction of toxicity of Cu^{2+} ions (at LC_{50}) to *Artemia salina* was observed in all tests in which AHS was present, regardless of the concentration.

Table 3. Mortality rate of *Artemia salina* exposed to copper ions (19 mg L^{-1}) in the presence of aquatic humic substances (AHS) at different concentrations

Concentration of AHS (mg L^{-1})	Mortality rate (%) (n=48)	Standard deviation
25	34.2	15.9
50	19.7	12.5
75	15.2	8.5
100	19.6	10.7

Figure 2A shows the application of the Fisher's LSD test to the results of the mortality rate of *Artemia salina* exposed to Cu^{2+} (19 mg L^{-1}) in the presence of different AHS concentrations. The first bar represents the mortality rate for Cu^{2+} at LC_{50} when AHS is absent. Figure 2B compares the reduction of toxicity of different samples as AHS concentrations increased.

Table 4 shows the results obtained from the toxicity assays using Cu^{2+} ions at 30 mg L^{-1} in the presence of AHS concentrations varying from 25 to 100 mg L^{-1}. In these tests, 100% of the test organisms died at the highest AHS concentration.

Figure 3A shows the application of the Fisher's LSD test to the results of the mortality rate of *Artemia salina* exposed to Cu^{2+} (30 mg L^{-1}) in the presence of AHS at different concentrations. The first bar represents the mortality rate for Cu^{2+} ions when AHS is absent. Figure 3B shows the decrease of toxicity of Cu^{2+} in the presence of different AHS concentrations in comparison to the test without AHS.

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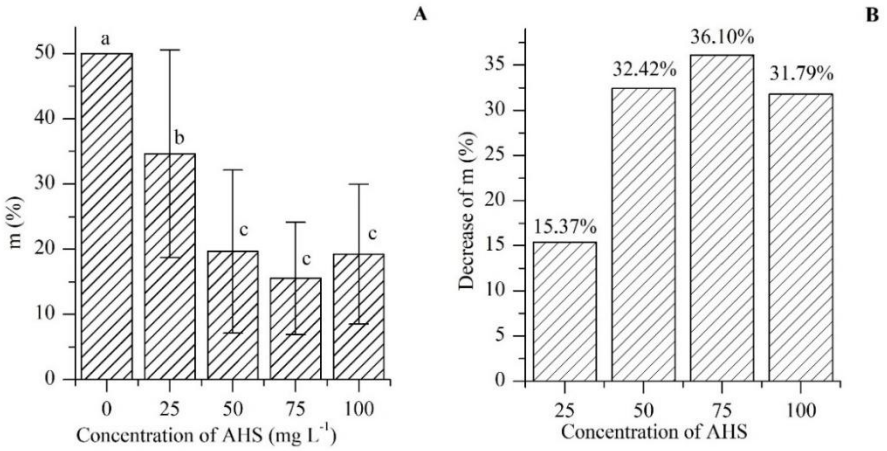

Figure 2. Reduction of toxicity of Cu^{2+} (19 mg L^{-1}) in the presence of aquatic humic substances (AHS) at different concentrations. A) Mortality rate (m) of *Artemia salina* for different AHS concentrations. B) Decrease in the mortality rate of *Artemia salina* for each AHS concentration tested in comparison to the test without AHS. Small letters on the bars stand for significative variations determined by the Fisher's LSD test with p-value<0.05.

Table 4. Mortality rate of *Artemia salina* exposed to copper ions (30 mg L^{-1}) in the presence of aquatic humic substances (AHS) at different concentrations

Concentration of AHS (mg L^{-1})	Mortality rate (%) (n=48)	Standard deviation
25	63.0	13.4
50	64.0	15.0
75	64.0	15.0
100	100.0	0.0

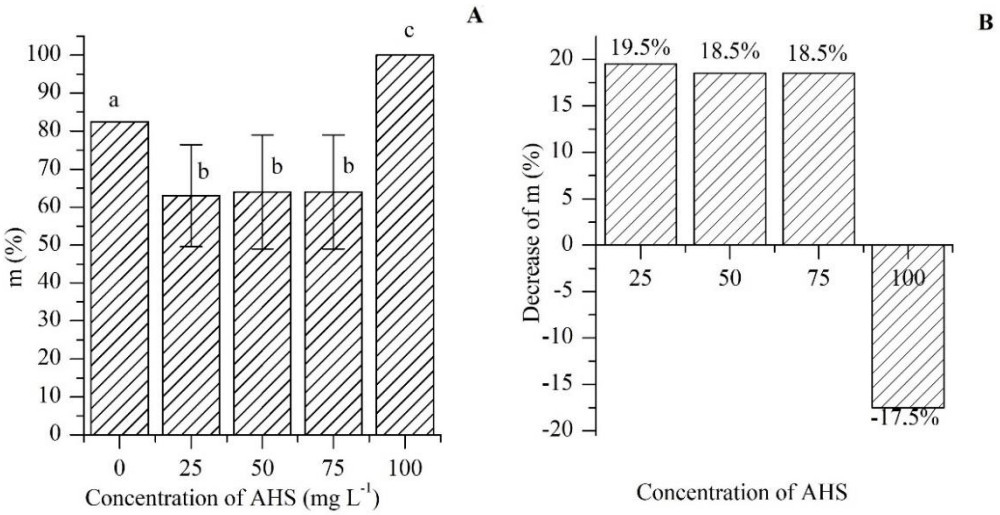

Figure 3. Reduction of toxicity of Cu^{2+} (30 mg L^{-1}) in the presence of aquatic humic substances (AHS) at different concentrations. A) Mortality rate (m) of *Artemia salina* for different AHS concentrations. B) Decrease in the mortality rate of *Artemia salina* for each AHS concentration tested in comparison to the test without AHS. Small letters on the bars stand for significative variations determined by the Fisher's LSD test with p-value<0.05.

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Potential of HA and FA at different ratios and concentrations in the reduction of acute toxicity of Cu^{2+} ions to *Artemia salina*

Figure 4 shows the mortality rates of *Artemia salina* in the presence of different concentrations of AHS with varying HA and FA ratios.

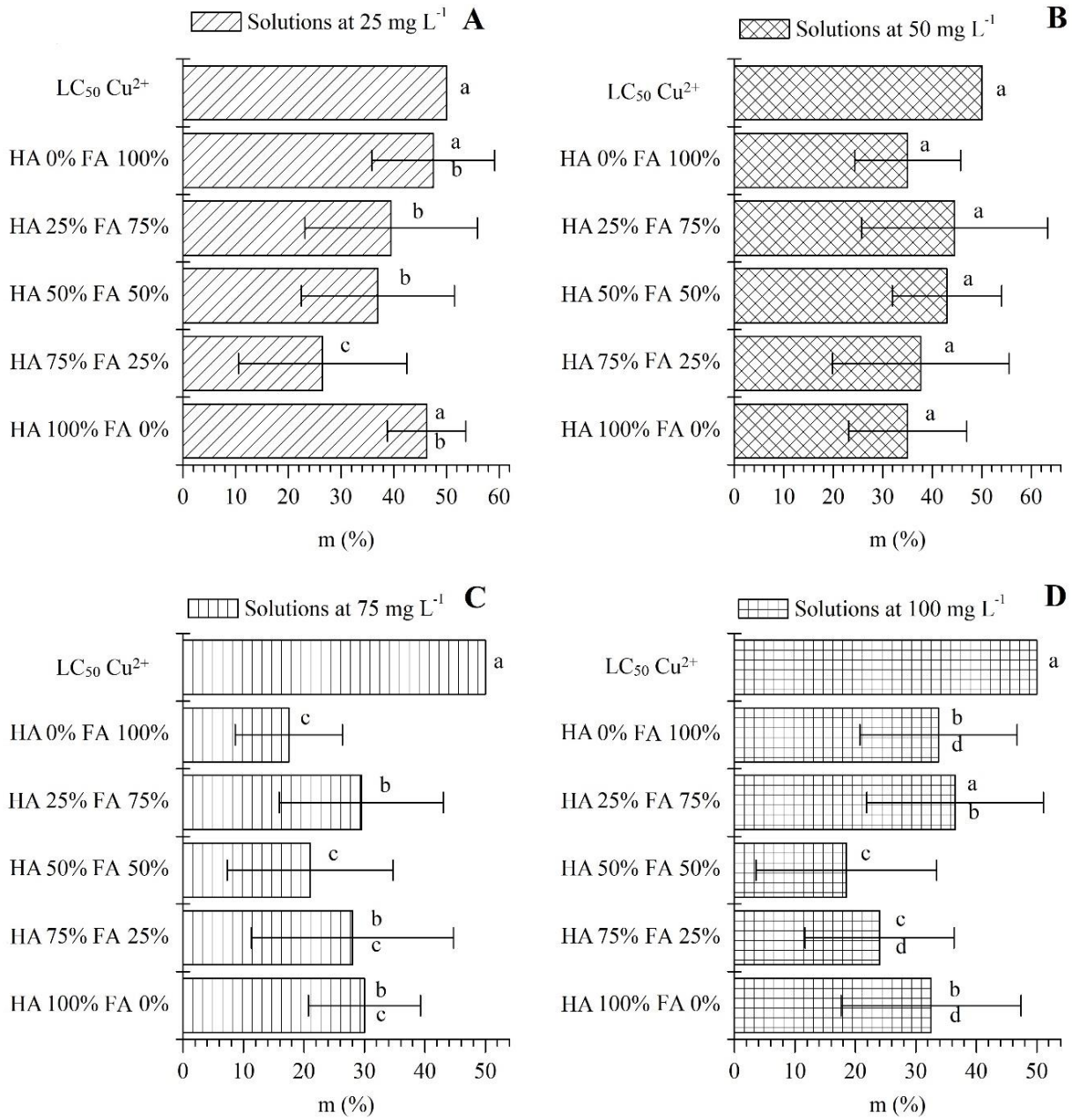

Figure 4. Decrease in the mortality rates (m) of *Artemia salina* exposed to Cu^{2+} in the presence of aquatic humic substances (AHS) at different concentrations, with varying ratios of humic acids (HA) and fulvic acids (FA), in comparison to the test without AHS. A) Solutions of AHS at 25 mg L^{-1}; B) Solutions of AHS at 50 mg L^{-1}; C) Solutions of AHS at 75 mg L^{-1}; D) Solutions of AHS at 100 mg L^{-1}. Small letters on the bars stand for significative variations determined by the Fisher's test with p-value<0.05.

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4. Discussion

Acute toxicity of Cu^{2+} ions

The results (Table 1) showed that from 19 mg L^{-1} onwards, the concentration of Cu^{2+} was lethal for more than 50% of the test organisms. Therefore, this was the LC_{50} used as reference in the tests to assess the decrease in the mortality rate of *Artemia salina* in the presence of AHS.

Acute toxicity of AHS

AHS LC_{50} value could not be determined precisely due to insufficient material available for further tests. However, the results (Table 2) suggested that the AHS LC_{50} for *Artemia salina* was probably between 400 mg L^{-1} and 600 mg L^{-1}, meaning that concentrations below 400 mg L^{-1} could be safely used, since they would not alter the mortality rate of the test organisms. In addition, concentrations higher than 50 mg L^{-1} of AHS are not typical of rivers such as Rio Negro, whose natural AHS concentrations normally range from 15 to 25 mg L^{-1} (Rocha *et al.*, 2003; Oliveira *et al.*, 2007; Araújo, Rosa, and Rocha 2002). Thus, the maximum AHS concentration used in all acute toxicity assays was of 100 mg L^{-1}.

Assessment of the reduction of Cu^{2+} acute toxicity to *Artemia salina* in the presence of AHS

The mortality rate of *Artemia salina* exposed to Cu^{2+} at 19 mg L^{-1} (LC_{50}) in the presence of AHS was lower than in the tests performed without AHS (Table 3 and Figure 2), thus supporting the potential of AHS in reducing the toxicity of Cu^{2+} ions. In the assays, the mortality rate reduced 15.37% in the presence of AHS at 25 mg L^{-1} and decreased nearly two times (to 32.42%) when the concentration of AHS doubled. In theory, the more amount of AHS is in the environment, the more sites Cu^{2+} ions have available to bind, which would reduce their presence and lethality to the organisms (Stevenson, 1994). However, as AHS concentrations increased in the assays, the mortality rate did not decrease significantly. A possible explanation might be the exchange of Cu^{2+} ions by other metals already present in the solution and more toxic, such as Hg^{2+} (Burba, Rocha, and Klockow, 1994). This observation indicates that the continuous increase of AHS concentration does not always mean a higher potential to reduce toxicity. Another reason may be the changes in the AHS molecular conformation, which becomes more densely coiled at high concentrations, thus reducing the availability of binding sites for Cu^{2+} (Swift, 1989).

When a higher Cu^{2+} concentration (30 mg L^{-1}) was tested, its acute toxicity to *Artemia salina* was less pronounced than in the tests at 19 mg L^{-1}. The mortality rate decreased from 85% to a rate between 19.5% and 18.5% in the presence of AHS at concentrations varying from 25.50 mg L^{-1} to 75 mg L^{-1}, which is not a significant reduction of the mortality rate for this AHS concentration range. This result was similar to the one found when Cu^{2+} at 19 mg L^{-1} was tested in the presence of AHS concentration ranging from 50 mg L^{-1} to 100 mg L^{-1}. The assays in the presence of AHS at 100 mg L^{-1} (and at 30 mg L^{-1}) showed a surprising increase in the mortality rate, once all the testing organisms died. This fact may also be explained by the changes that occur in the conformation of AHS molecules when they are highly concentrated (Hayes, 1985). Furthermore, as AHS concentrations heighten, other toxic metal ions existing in their molecules may be released, which increases the mortality of *Artemia salina*.

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Assessment of the potential of HA and FA at different ratios and concentrations in the reduction of acute toxicity of Cu^{2+} ions to *Artemia salina*

Decrease in the Cu^{2+} acute toxicity to the test organisms was not observed in the assays performed in the presence of isolated HA 100% and FA 100% (25 mg L^{-1}). This behaviour suggests that the isolated fractions HA and FA are not good at reducing acute toxicity. Reduction in the acute toxicity was only observed in the tests of HA and FA when these were combined, as the mortality of *Artemia salina* decreased significantly. These findings suggest that the simultaneous presence of HA and FA is responsible for reducing the acute toxicity of Cu^{2+} in the presence of AHS (25 mg L^{-1}), regardless of the HA and FA ratios. This may be due to the difference in size and structure of HA and FA, whose combination may alter AHS conformation, therefore forming stabler complexes. The results also indicate that the higher the HA ratios (assays with AHS at 25 mg L^{-1}, Figure 4), the lower the acute toxicity of Cu^{2+} ions to the test organisms. Although the tests using FA and HA presented a high standard deviation, the results are useful to indicate trending effects in the acute toxicity of Cu^{2+} ions in the presence of AHS.

In the assays in which different concentrations of AHS and different ratios of HA and FA (except the one of AHS at 50 mg L^{-1}, Figure 4) were assessed, all solutions presented a significant decrease in the acute toxicity of Cu^{2+}. However, it was not possible to identify if the fractionated HA (100% HA) and FA (100% FA) could reduce Cu^{2+} acute toxicity to the same degree that the other ratios of these fractions did. The results did not demonstrate satisfactory accuracy, which significantly compromised statistical trends assessments. It probably occurred due to higher AHS concentrations, which caused conformational changes in the AHS molecules, reducing their binding sites. Furthermore, exchange of Cu^{2+} ions by other metals more toxic and already present in AHS would induce these ions to be released, increasing the mortality of *Artemia salina*. These results are compatible with the ones found in the assays of AHS at varying concentrations (same ratio, Figure 3), whose data when concentration of AHS was higher than 25 mg L^{-1} were also more difficult to assess.

5. Conclusions

The acute toxicological assays performed in this investigation pointed out that the LC_{50} of Cu^{2+} ions to *Artemia salina* is 19 mg L^{-1} and concentrations of AHS below 400 mg L^{-1} can be used without altering the mortality rate of the test organisms. However, further studies are necessary to define the exact AHS LC_{50}, value, which was not determined in the current study due to insufficient amount of AHS for performing extra tests. The results indicated that the LC_{50} of AHS to *Artemia salina* lies in the concentration range of 400 to 600 mg L^{-1} of AHS. This study revealed that the presence of AHS (not fractionated and at concentrations up to 100 mg L^{-1}) reduced the mortality rate of *Artemia salina*. The assays of varying ratios of HA and FA at 25 mg L^{-1} showed a trend of reduced acute toxicity as HA increased. Furthermore, the isolated fractions HA and FA did not reduce Cu^{2+} acute toxicity to *Artemia salina* as much as they did when combined, either when their fraction ratio were varied or when they formed AHS solutions. These results support that AHS can trap metal ions more effectively when the variety of macromolecules is greater. Additionally, at higher AHS concentrations, statistical assessment was more difficult to perform, once results were less accurate. This was probably related to the conformational changes of AHS at higher concentrations or due to the exchange of Cu^{2+} ions by

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other existing metal ions in the AHS structure.

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