Ciências Exatas

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

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Resumo

Nesta investigação, o potencial das substâncias húmicas aquáticas (SHA) para diminuir a toxicidade dos íons cobre (Cu^{2+}) foi avaliado utilizando o organismo teste Artemia (*Branchipus stagnalis*). 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 foram primeiramente determinadas. Diferentes quantidades de SHA foram misturadas com íons Cu^{2+} na CL_{50}, a fim de estimar a taxa de mortalidade de Artemia em comparação com os testes sem SHA. As frações SHA, ácidos húmicos (AH) e ácidos fúlvicos (AF) em diferentes proporções também foram testadas. Os resultados mostraram que a CL_{50} de Cu^{2+} foi de 19 mg L^{-1} e para SHA foi entre 400 e 600 mg L^{-1}. Os resultados sugerem que a SHA tem maior poder redutor de toxicidade que suas frações de ácido húmico e fúlvico separadas. Os ensaios com SHA mostraram uma redução de até 40 % na mortalidade das Artemias. Já os ensaios com AH e AF apresentaram uma diminuição de até 20 % na taxa de mortalidade. Nos experimentos com diferentes proporções de AH e AF, os resultados sugerem maior redução da toxicidade de Cu^{2+} quando a proporção de AH é maior.

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

Lethal concentration (LC_{50}) and potential of aquatic humic substances from Rio Negro (Brazil) to decrease the toxicity of Cu^{2+} ions to Artemia salina. In this investigation, the potential of aquatic humic substances (AHS) to decrease the toxicity of copper (Cu^{2+}) ions was evaluated using the test organism Artemia salina (Branchipus stagnalis). The LC_{50} (concentration that kills 50% of a population of a test organism) values of Cu^{2+} and AHS to the test organism were firstly determined. Different amounts of AHS were mixed with Cu^{2+} ions at LC_{50} in order to estimate the mortality rate of Artemia salina compared to the tests without AHS. The AHS fractions humic acids

[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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(HA) and fulvic acids (FA) in different proportions were also tested. 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 findings of this study suggest that AHS reduce the toxicity of Cu^{2+} to a greater extent than their fractions HA and FA individually. The trials with AHS showed a reduction of up to 40 % in the mortality of Artemia salina. On the other hand, HA and FA presented a decrease up to 20 % in the mortality rate. The results of experiments with different proportions of HA and FA indicated that there is greater reduction of the toxicity of Cu^{2+} when the proportion 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 decomposition of plants and animals, comprising most of the dissolved organic carbon in natural waters (McDonald *et al*., 2004). They affect significantly 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) because of their strong affinity for each other. 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 about how changes in the concentration of AHS and their major fractions humic acids (HA) and fulvic acids (FA) affect the toxicity reduction of potentially toxic metals (Li *et al.*, 2018; Zhang *et al*., 2019).

The transport and fate of metals are affected by different factors as precipitation/dissolution, oxidation/reduction, adsorption/desorption and complexation (Stumm and Morgan, 1995). Then, all these processes must be considered in order to understand the behaviour of potentially toxic metals in natural systems, which makes necessary the characterisation of their different chemical species, including AHS-metal complexes. The environmental quality standards in 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 depends on complexing or chelating agents in solution (Väänänen *et al.*, 2018).

The presence of AHS can change biological adsorption processes of lots 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 the toxicity is the lethal concentration (LC_{50}) (Stephan, 1977). The LC_{50} is the concentration of a chemical that kills 50% of a population of a test organism (Boyd, 2005).

Artemia salina (*Branchipus stagnalis*) is a test organism generally used for determination of the LC_{50} of chemicals, especially in the pharmaceutical industry (Arcanjo *et al*., 2012; Cavalcante *et al*., 2000; Pimentel *et al*., 2011). The Artemia or brine shrimp (popular name) is a small crustacean of the order Anostraca and

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unique genus of *Artemidae* family. These organisms have high resistance to adverse conditions and can live in water with high levels of salinity (Dumitrascu, 2011). Artemias are often used in ecotoxicological assays because they are easily acquired in markets in form of cysts at a low cost. In addition, the organisms have rapid hatching and the ecotoxicity testing protocols (both 24 h) with this brine shrimp found in literature provide fast results (Rajabi *et al*., 2015).

The main purpose of this study is to evaluate how AHS and their fractions HA and FA contribute to decrease the toxicity of Cu^{2+}. A series of acute toxicity assays were performed using the lethal concentration (LC_{50}) of Cu^{2+} to the test organism Artemia Salina in the presence of AHS at different amounts and the fractions HA and FA in different proportions (since the proportions of HA and FA can be associated to the formation of humus). These experiments sought to understand how the concentration and composition of these substances change the reduction effect of acute toxicity.

2. Materials and methods

Hatching of Artemia salina cists

Dormant cysts of the Artemia salina (*Branchipus stagnalis*) were purchased from an aquarium shop and 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 (15 W fluorescent lamp, 6400 K) and continuous aeration maintained by an air pump (Boyu U-02800) for 24 h (Figure 1) (Silva *et al.*, 2010; Vanhaecke *et al*., 1981). After the incubation time, the hatched Artemia salina nauplii were separated from the non-hatched cysts.

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

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

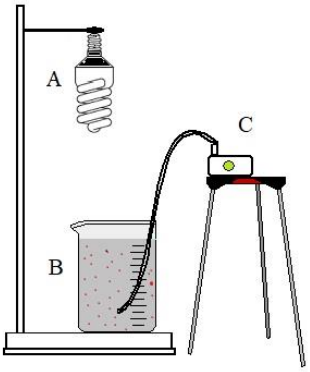

Figure 1. Schematic representation of apparatus used for hatching of Artemia salina cysts. A) 15 W fluorescent lamp, 6400 K; B) Beaker (200 mL); C) air compressor pump.

The extraction of AHS was performed based on the procedure recommended by the International Humic Substances Society (2018), using acrylic ester resin XAD-8 to adsorb the AHS in acidic medium (river water acidified until pH<2 using hydrochloric acid (HCl)) and desorb them 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 gave a negative chloride test with silver nitrate ($AgNO_3$).

Half of the dialysed extract was reserved for fractionation between the fractions HA and FA. The other half was lyophilised and stored in amber tubes until 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 and lyophilised afterwards (Thurman and Malcolm, 1981).

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Preparation of solutions

A synthetic seawater solution (4 g L^{-1}) was prepared using sea salt (Red Sea Salt), whose composition was magnesium (Mg, 1230 ppm), potassium (K, 403 ppm), sulphur (S, 889 ppm) and its 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 for using in the acute toxicity assays, where it was diluted to the target concentrations (5 to 50 mg L^{-1}).

The powder of the AHS extracted was diluted in the synthetic seawater to 600 mg L^{-1} AHS, following a pH adjustment to 5 (pH of Rio Negro River). This AHS was 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 Artemia salina nauplii, where 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, where 1 mL of the 4 g L^{-1} sea salt solution was added. An aliquot (depending on the target concentration) of the 0.57 g L^{-1} $Cu(NO_3)_2$ solution was added subsequently in the tests for investigating the acute toxicity of Cu^{2+} and influence of AHS. A pre-determined mass of AHS was also added at some tests into each cell, which had their volumes completed with the sea salt solution. All cell culture plates were incubated at 25 °C for 24 h and the surviving larvae were counted afterwards using a magnifying glass. The culture plates were gently shaken, and the larvae were considered dead if they did not exhibit movement during 10 s of observation. The nauplius were not fed during the tests because they consume the energy reserves stored in the cysts in their first 24 h (Sorgeloos, 1978; Briski *et al.*, 2008).

The mortality rate (m) of Artemia salina was calculated using to the 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). The average of the mortality percentage between cells of two culture plates was used to calculate m, resulting in a sample size of 48.

Acute toxicity of Cu ions to Artemia salina

The first set of experiments used copper nitrate ($Cu(NO_3)_2$) at concentration between 5 to 50 mg L^{-1} to determine the lethal concentration (LC_{50}, defined as concentration of solvent/substance that kills 50 % of a group of the test organisms according to Vanhaecke (1981) of Cu^{2+} ions to Artemia salina. The LC_{50} of Cu^{2+} ions was used in the experiments to investigate the effects of AHS and their fractions to reduce 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 can be used in the toxicity assays without causing acute effects in the organisms.

Evaluation of the decrease of acute toxicity of Cu^{2+} ions to Artemia salina by AHS

The potential of AHS and their fractions HA and FA to reduce the acute toxicity of Cu^{2+} ions to Artemia salina was analysed 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 e 100 mg L^{-1}).

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Evaluation of the potential of HA and FA at different concentrations and proportions to decrease the acute toxicity of Cu^{2+} ions to Artemia salina

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

Statistical analysis

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

3. Results

Acute toxicity of Cu ions to Artemia salina

The results obtained from the tests with copper ion solutions at concentrations from 0 to 50 mg L^{-1} after exposure of 24 h to Artemia salina are summarised in Table 1

Acute toxicity of AHS to Artemia salina

The results of the acute toxicity assays of AHS at different concentrations to Artemia salina are shown in Table 2. The LC_{50} of AHS to Artemia salina could not be precisely determined from these experiments.

Evaluation of the decrease of acute toxicity of Cu^{2+} to Artemia salina by AHS

Table 3 shows the results obtained from toxicity assays using concentrations from 25 to 100 mg L^{-1} of AHS mixed with Cu^{2+} ions at LC_{50} (19 mg L^{-1}). Tests with the lowest AHS concentration presented mortality rates ca. 2 times higher than other tests using higher amounts of AHS. However, the reduction of toxicity of Cu^{2+} ions to Artemia salina was observed for all tests at LC_{50} of Cu^{2+} ions.

The Figure 2A shows the application of Fisher's LSD test to the results the mortality index of Artemia salina to Cu^{2+} (19 mg L^{-1}) at different AHS concentrations. The first bar represents the mortality index for Cu^{2+} at LC_{50} without AHS. Figure 2B compares the reduction of the toxicity of different samples when AHS concentraion incread.

The results of the toxicity assays using concentrations from 25 to 100 mg L^{-1} of AHS mixed with Cu^{2+} ions at 30 mg L^{-1} are shown in Table 4. In these tests, 100 % of the organism died at the highest tested concentration of AHS.

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

Evaluation of the potential of HA and FA at different concentrations and proportions to decrease the acute toxicity of Cu^{2+} ions to Artemia salina

Figure 4 shows the mortality rates to Artemia salina after addition of different concentrations of AHS with different proportions of HA and FA.

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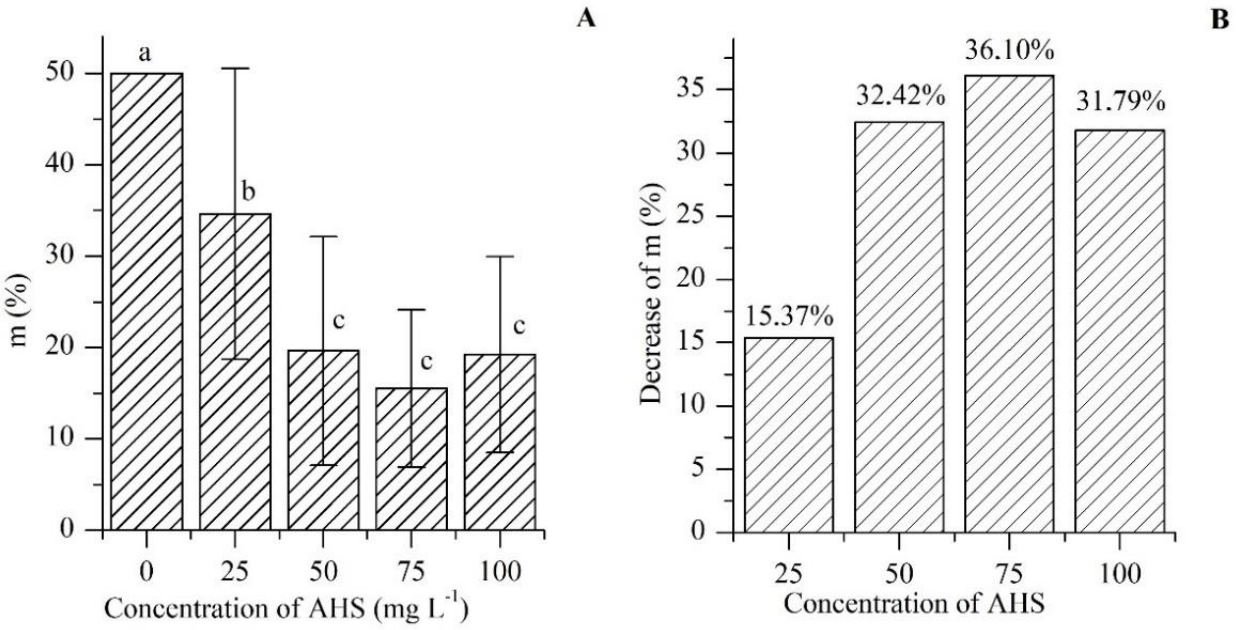

Figure 2. Evaluation of the reduction of toxicity of Cu (19 mg L^{-1}) in the presence of aquatic humic substances (AHS) at different concentrations. A) Mortality rate (m) at different concentrations of AHS. B) Decrease of mortality rate of Artemia salina at each tested AHS concentration in comparison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

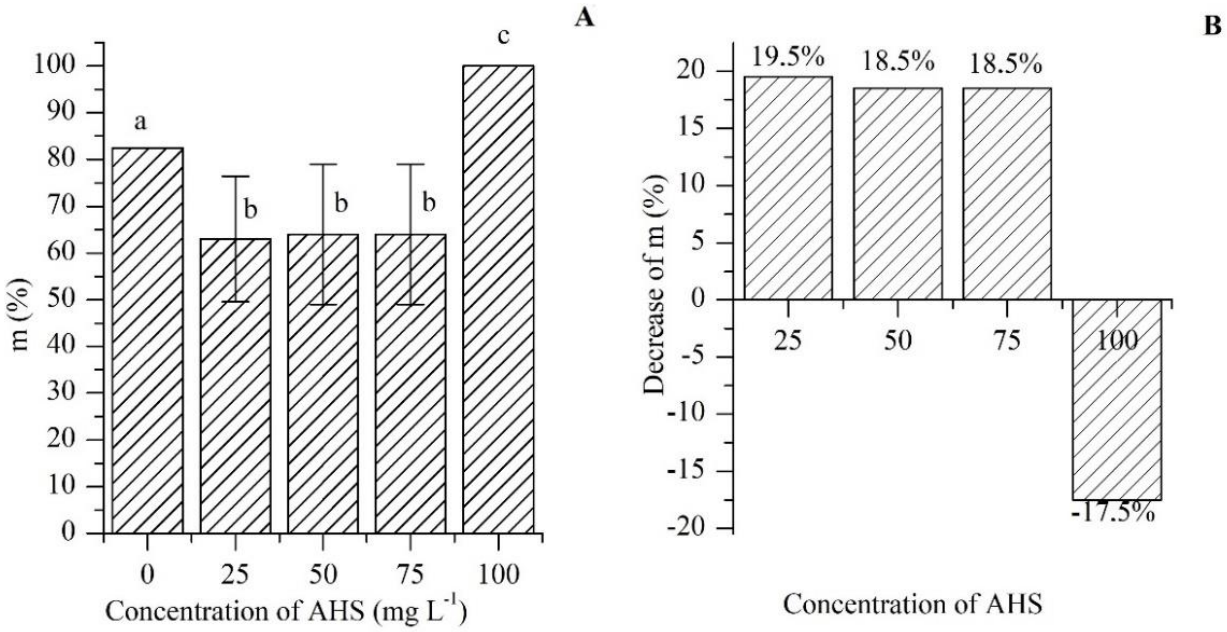

Figure 3. Evaluation of the reduction of toxicity of Cu^{2+} (30 mg L^{-1}) in the presence of aquatic humic substances (AHS) at different concentrations. A) Mortality rate (m) at different concentrations of AHS. B) Decrease of mortality rate of Artemia salina at each tested AHS concentration in comparison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

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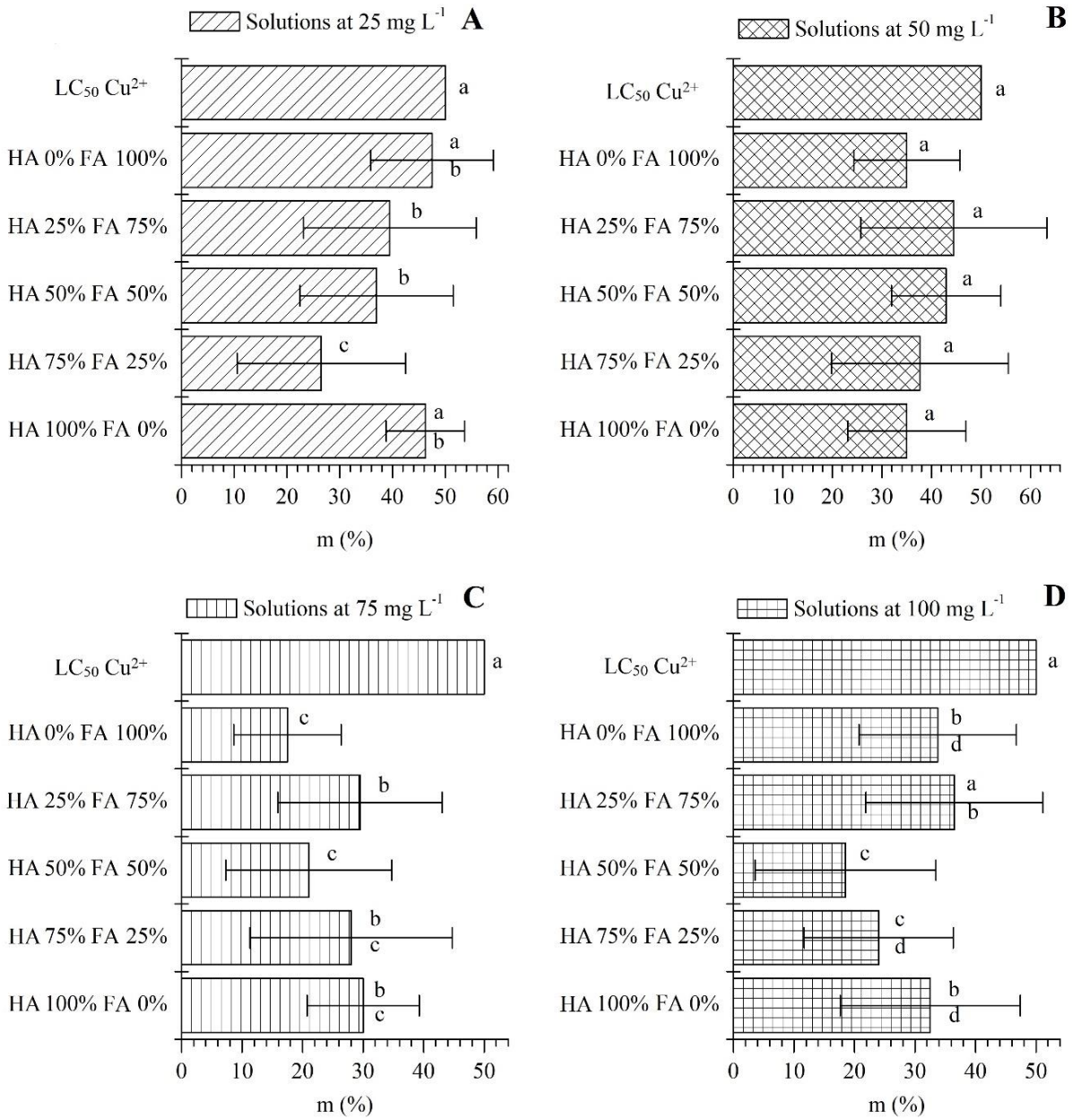

Figure 4. Evaluation of the decrease of mortality rates (m) of Artemia salina to Cu^{2+} in the presence of different concentrations of aquatic humic substances (AHS) with different proportions of humic acids (HA) and fulvic acids (FA) in comparison to the test without AHS. A) Solutions at 25 mg L^{-1}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

4. Discussion

Acute toxicity of Cu^{2+} ions

The results (Table 1) showed that Cu^{2+} concentrations higher than 19 mg L^{-1} were lethal for more than 50 % of the test organisms. Therefore, this concentration was the LC_{50}, used as reference in the tests carried out to evaluate the decrease of the mortality rate in the presence of AHS.

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

Concentration of Cu (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

Acute toxicity of AHS

The LC_{50} of AHS could not precisely be determined because of the low mass of material available for further tests. However, the results (Table 2) suggested that the LC_{50} of AHS is probably between 400 mg L^{-1} and 600 mg L^{-1}. Therefore, concentrations below 400 mg L^{-1} can be safely used without affecting the mortality rate of the test organisms. In addition, concentrations higher than 50 mg L^{-1} of AHS are not typical from rivers like Rio Negro River, 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). Then, the maximum AHS concentration used in all acute toxicity assays was 100 mg L^{-1} since higher concentrations are not typical from rivers and the mortality rate of Artemia salina would not be affected in the experiments for investigating the decrease of Cu^{2+} toxicity in the presence of AHS.

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

Evaluation of the decrease of acute toxicity of Cu^{2+} ions to Artemia salina by AHS

The mortality rate of Artemia salina in the tests with Cu^{2+} at 19 mg L^{-1} (LC_{50}) in the presence of AHS was lower than in tests without AHS (Table 3 and Figure 2), supporting the potential of AHS to reduce the toxicity of Cu^{2+} ions. In the trials, the mortality rate reduced 15.37 % at 25 mg L^{-1} of AHS and decreased nearly two times (to 32.42 %) when the concentration of AHS doubled. On the other hand, the mortality rate did not decrease significantly at higher AHS concentrations, although more binding sites would be theoretically available, resulting in a low availability of Cu^{2+} ions to the organisms (Stevenson, 1994). A possible explanation for this might be the exchange of Cu^{2+} ions by other ions

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(Burba, Rocha, and Klockow, 1994), indicating that the continuous increase of AHS concentration does not always mean a higher capacity to decrease the toxicity. Some possible explanations for this might be the exchange of Cu^{2+} by other more toxic ions such as Hg^{2+} already present in the solution or changes in the conformation of AHS molecules, which become more densely coiled at high concentrations, decreasing the availability if binding sites to Cu^{2+} (Swift, 1989).

Table 3. Mortally rate of Artemia salina to copper ions (19 mg L^{-1}) at different concentrations of aquatic humic substances (AHS).

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

Table 4. Mortally rate of Artemia salina to copper ions (30 mg L^{-1}) at different concentrations of aquatic humic substances (AHS).

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

When a higher Cu^{2+} concentration (30 mg L^{-1}) was tested, the acute toxicity of the metal to Artemia salina was less pronounced than in the tests at 19 mg L^{-1}. At all tested concentrations of AHS, the mortality rate was above the mortality rate recorded for LC_{50}. The mortality rate decreased up to 23.6 % in the presence of AHS at concentrations of 25, 50 e 75 mg L^{-1}. However, the results showed that there was no decrease of toxicity when AHS concentration increased from 25 to 50 mg L^{-1} and from 50 to 75 mg L^{-1} (as happened for the tests with 19 mg L^{-1} of Cu^{2+}). In the trials in the presence of 100 mg L^{-1} of AHS, there was surprisingly an increase of the mortality rate, since all test organisms died. This fact may be also explained by changes in the conformation of AHS molecules at high concentrations (Hayes, 1985). Furthermore, other toxic metal ions already present in AHS molecules can be released at higher concentrations, increasing the mortality of Artemia salina.

Evaluation of the potential of HA and FA at different concentrations and proportions to decrease the acute toxicity of Cu^{2+} ions to Artemia salina

It was not observed a decrease of Cu^{2+} acute toxicity to the test organisms in the trials performed in the presence of isolated HA 100 % and FA 100 % (25 mg L^{-1}). This behaviour suggests that isolated HA and FA are not good at reducing the acute toxicity. A reduction in the acute toxicity was observed just in the trials with mixed HA and FA, where there the mortality of Artemia salina decreased significantly. These findings suggest that the simultaneous presence of HA and FA is responsible for decreasing the acute toxicity of Cu at concentration of 25 mg L^{-1}, independent of the proportion of HA and FA. It

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may be due to the difference in size and structure of HA and FA, which can reduce free space in the structure of AHS when mixed, forming therefore more stable complexes. The results also indicate that the higher the proportions of HA (experiments in the presence of 25 mg L^{-1} of AHS, Figure 4), the lower the acute toxicity of Cu^{2+} ions to the test organisms. Although these experiments using FA and HA presented a high standard deviation, the results are useful to indicate trends of effects of the presence of AHS in the acute toxicity to Cu^{2+} ions.

In the other trials using different concentrations of AHS and proportions of HA and FA (except the one in the presence of 50 mg L^{-1} of AHS, Figure 4), all solutions presented a significant decrease in the acute toxicity of Cu^{2+}. However, it was not possible to identify if the isolated HA (100 % HA) and FA (100 % FA) reduced the acute toxicity to Cu^{2+} when compared to other proportions of these fractions. The results are dispersed, compromising statistical trends evaluation. The dispersion is probably due to the higher AHS concentrations, which caused conformational changes in AHS molecules, reducing their binding sites. Furthermore, exchange of Cu ions by other more toxic ions present in the AHS would cause release of these ions and therefore increase the mortality of Artemia salina. These results agree to the data of the experiments varying the concentrations of AHS (same proportion, Figure 3), where the evaluation of data at AHS concentrations higher than 25 mg L^{-1} was also more difficult to analyse.

5. Conclusions

In this investigation, the acute toxicological assays 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 affecting the mortality rate of the test organisms. However, further studies are necessary to define the exact LC_{50} of AHS, which was not determined in the current investigation because of insufficient amounts 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 has revealed that the presence of AHS without fractionation (at concentrations up to 100 mg L^{-1}) decreased the mortality rate of Artemia salina. The trials using different proportions of HA and FA at 25 mg L^{-1} showed a trend of decreasing the acute toxicity when the proportion of HA increases. Furthermore, the isolated fractions HA and FA did not present reduction of acute toxicity when compared to mixed HA and FA in different proportions and AHS solutions. This suggests that the isolated fractions are not efficient in reducing the toxicity as when both fractions are simultaneously present. These results support that AHS can trap ions more effectively when there is higher variety of macromolecules. The results of the tests at higher AHS concentrations presented a higher dispersion, turning the statistical evaluation more difficult. This dispersion is probably linked to conformational changes of AHS when present at higher concentrations or exchange of Cu^{2+} by other ions already present in AHS structure.

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Disclosure statement

This paper contains original research and has not been submitted or published elsewhere. The authors and reviewers did not report any competing financial interests or personal relationships that could have appeared to influence the work. Then, the journal Scientia Amazonia holds copyright, has approval and permission of the authors to publish this paper in electronic media.

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