CARLA ROLIM FERRARI

Avaliação de Efluentes Radioativos da Unidade de Tratamento de Minério das Indústrias Nucleares do Brasil e de Bacia de Drenagem sob sua influência, com Ênfase na Caracterização Químíca e Ecotoxicológica

Tese apresentada ao Programa de Pós-Graduação Interunida- des em Biotecnologia USP/ Instituto Butantan/IPT, para obtenção do Título de Doutor em Biotecnologia

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Certificamos que o Protocolo CEP-ICB Nº **666/14** referente ao projeto intitulado: "*Avaliação ecotoxicológica de efluentes radioativos da Unidade de Tratamento de Minério das Indústrias Nucleares do Brasil (UTM/INB) e de suas bacias de drenagem, com ênfase na toxicidade de metais para organismos planctônicos*" sob a responsabilidade de **Carla Rolim Ferrari,** foi analisado na presente data pela CEUA - COMISSÃO DE ÉTICA NO USO DE ANIMAIS e pela CEPSH- COMISSÃO DE ÉTICA EM PESQUISA COM SERES HUMANOS, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da lei 11.794 de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº466 de 2012.

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PROF. DR. PAOLO M.A ZANOTTO
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"Na vida tudo tem seu apogeu e seu declínio. É natural que seja assim; todavia, quando tudo parece convergir para o que supomos o fim, eis que a vida resurge triunfante e bela...Novas folhas, novas flores, na indefinida benção do recomeço" Chico Xavier

RESUMO

FERRARI, C. R. **Avaliação de Efluentes Radioativos da Unidade de Tratamento de Minério das Indústrias Nucleares do Brasil e de Bacia de Drenagem sob sua influência, com Ênfase na Caracterização Químíca e Ecotoxicológica.** 2017. 115 f. Tese (Doutorado em Biotecnologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2017.

A primeira mineração de urânio do Brasil está localizada nas dependências da Unidade de Tratamento de Minérios das Indústrias Nucleares do Brasil (UTM/INB). Atualmente, essa área mineradora encontra-se em fase de descomissionamento e a drenagem ácida de mina (DAM) constitui o principal passivo ambiental desse site, devido ao grande volume de efluentes radioativos gerados, os quais após tratamento são lançados na represa das Antas (RA). Neste contexto, o presente estudo avaliou a qualidade da água através da caracterização química e ecotoxicológica em amostras de efluente *in natura* (Cava da Mina)e efluente tratado (P41-S) procedente da UTM/INB, bem como em amostras de água da RA, sob influência dessa mineradora. Adicionalmente testes de toxicidade aguda com os metais urânio e manganês também foram realizados, além da determinação da especiação química do urânio e sua relação com a toxicidade para dafinídeos. De acordo com os resultados amostras do efluente *in natura* e tratado apresentaram maiores concentrações de espécies químicas, sendo consideradas agudamente tóxicas para os dafinídeos. Tais resultados indicaram que o sistema de tratamento do efluente *in natura* realizado pela mineradora foi impróprio e ineficiente. Em novembro de 2014 na RA valores de dureza extremamente elevados (543,55 mg L^{-1}) indicaram efeito protetor para os dafínideos ($P<0,05$), quando maiores concentrações dos metais F^- (4,5 mg L^{-1}) U (0,082 mg L^{-1}), Mn (1,125 mg L^{-1}) e Al (1,55 mg L^{-1}) foram registradas. O metal Mn apresentou potencial de causar toxicidade, uma vez que concentrações de Mn registradas no presente estudo e na literatura estiveram acima do valor de CE_{50} (5,93 mg L^{-1} Mn) registrado para *C. silvestrii*. Para U, mudanças nos valores de pH nos ensaios de *D.magna* estiveram associadas a alterações na especiação desse metal, com maiores concentrações das espécies potencialmente tóxicas para a biota, ou seja, UO_2^{2+} e UO_2OH^+ sendo registradas em pH 7.Também a espécie nativa *Ceriodaphnia silvestrii* mostrou ser muito mais sensível ao metal urânio, quando comparada a *D. magna*, de acordo com os valores de CE_{50} registrados que foram: 0,07 e 0,56 mg L^{-1} U, respectivamente.

Palavras chave: Mineração de urânio. Efluentes radioativos. Drenagem ácida de mina Caracterização química e ecotoxicológica. Bioindicadores.

ABSTRACT

FERRARI, C. R. **Evaluation of the radioactive effluents from the Mineral Treatment Unit of the Brazilian Nuclear Industries and the Drainage Basin under their influence, with emphasis on the chemical and ecotoxicological characterization.** 2017. 115 p. Doctorate Thesis (Biotechnology) – Institute of Biomedical Science, University of São Paulo, São Paulo, 2017.

The first uranium mine in Brazil is located in the grounds of the Ore Treatment Unit of the Brazilian Nuclear Industries (UTM/INB). Currently this mining area is in the decommissioning phase and acid mine drainage (AMD) constitutes the main environmental liability of this site due to the great volume of radioactive effluent generated. The acid effluent is treated by physicochemical processes and released into the Antas reservoir. Thus, by way of ecotoxicological and chemical characterizations, the present study aimed to evaluate the effects of the discharge of the *in natura* (mine pit) and treated (P41-S) effluents proceeding from UTB/INB, into the Antas reservoir (CAB, P41-E, P14) under the influence of this mining company. In addition, acute toxicity tests with the metals of interest, uranium and manganese, were carried out, and the uranium speciation and its relationship with toxicity to daphnids determined. According to the results, samples of both the *in natura* and treated effluents presented high concentrations of some chemical species, and were considered acutely toxic to daphnids. These results indicate that the system used by the mining company to treat the *in natura* effluent was improper and inefficient. Also in November, 2014, Antas reservoir (AR) samples, showing very high hardness values (543.55 mg L^{-1}) showed positive correlation with the decrease in toxicity for daphnids ($P<0.05$), when higher concentrations of metals such as: fluoride (4.5 mg L^{-1}) uranium (0.082 mg L^{-1}), manganese (1.125 mg L^{-1}) and aluminum (1.55 mg L^{-1}) were registered. The metal manganese showed the potential to cause toxicity since the concentrations recorded in the present study and in the literature were above the EC_{50} value (5.93 mg L^{-1} Mn) registered for *C. silvestrii*. When the pH value was decreased from 8 to 7 the speciation of the metal uranium was shown to change, with greater concentrations of the species UO_2^{2+} and UO_2OH^{+}, considered potentially toxic to the biota, being registered at pH 7. Also the native species *Ceriodaphnia silvestrii* was shown to be much more sensitive to the metal uranium when compared to *D. magna*, according to the EC_{50} values recorded, which were 0.07 and 0.56 mg L^{-1} U, respectively.

Keywords: Uranium mining. Radioactive effluents. Acid mine drainage. Chemical and ecotoxicological characterization. Bioindicators.

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SUMMARY

THESIS PRESENTATION

The mode of presenting this Doctoral thesis in chapters favored publication of the results obtained in international scientific journals. Thus in relation to this form of presentation, some considerations should be taken into account. The thesis was divided in the following way: 1) Introduction and Justification; 2) General and Specific objectives; 3) Materials and Methods/Results and Discussion composed of four chapters; 5) General conclusions and Appendixes.

It should be highlighted that the Results obtained and Discussion were written in chapters in the form of scientific articles, as follows: **Chapter 3.1** Effects of the discharge of uranium mining effluents on water quality in the reservoir: an integrative limnological and ecotoxicological assessment; **Chapter 3.2** Acute toxicity of manganese for *Ceriodaphnia silvestrii* and *Daphnia magna* in bioassays and the potential toxicity of this metal in uranium mine effluents; **Chapter 3.3** An approach to the speciation and acute toxicity of uranium on the Daphnids *Daphnia magna* and *Ceriodaphnia silvestrii*; and **Chapter 3.4** Evaluation of the treatment system of the uranium mine effluent, from the chemical characterization and acute toxicity potential carried out using *Ceriodaphnia silvestrii* and *Daphnia magna*.

The chapters of the present thesis were written and formatted according to the norms of the scientific journals of interest, that is: **Chapter 3.1** - Scientific Reports, **Chapter 3.2** - Mine Water and the Environment; **Chapter 3.3** and **Chapter 3.4** - Archives of Contamination and Toxicology. Since the majority of scientific journals require that the papers be written in English, all the chapters were written in this language, in order to make it easier to submit them to the above cited international journals of interest.

The elaboration of the thesis in this format is justified by the fact that it makes the publication of the articles in scientific journals quicker and less onerous, although, due to the independence of each chapter in relation to the others, some repetitions are inevitable.

1 INTRODUCTION AND JUSTIFICATION

1.1 Contextualization of the proposed study

Mining activities have contributed significantly to the growth and development of various countries, including Brazil, since the importance of this sector for the Brazilian economy is significant, with emphasis on the production of iron, niobium, manganese, and aluminum (bauxite), amongst others (IBRAM, 2008). Although the production of uranium in Brazil is not significant in terms of volume, it is important to point out that it is an integral part of the nuclear fuel cycle, and is therefore considered to be strategic for the country. It should also be pointed out that Brazil belongs to a restricted group of countries that dominate the nuclear technology referring to the fuel cycle, as from extraction of the mineral up to the production of nuclear electricity.

Nevertheless the expansion and continuation of mining activities in Brazil and in the world depends on the incentive and compromise of the large mining companies with respect to the preservation and recovery of the explored environment, so as to conform to the legal demands of the control organs, such as: The Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA), The National Water Agency (ANA) and pertinent state and municipal organs such as The National Nuclear Energy Commission (CNEN), responsible for controlling and managing nuclear installations.

With respect to concerns about the impacts caused by mineral exploration, the question of water and water resources is frequently one of the most important conflicts involving mining and the society (REBOUÇAS; BRAGA; TUNDISI, 2006). According to Antunes et al. (2007), mining activities can involve great changes to the contour of the landscape, since they are related to chemical, physical and biological alterations to the environment explored.

In general, mining activities can cause significantly negative impacts to the environment, since in the majority of cases the development of such activities implies in: suppression of vegetation, soil exposure and erosion, alterations in the quality of the surface and subterranean water resources, in addition to causing air pollution, amongst other negative aspects associated with these activities (MECHI; SANCHES, 2010). Depending on the geological characteristics of the deposit of interest, some mining activities can also result in acid mine drainage (AMD), as well as generating mineral tailings and increasing the turbidity and silting of water bodies, which can directly affect the water resources in the region where they occur. AMD has been considered one of the main negative environmental impacts

resulting from mining activities, due to its dynamics and persistence (SANTOS; LADEIRA, 2011). Another negative aspect that can result from mining activities is related to the occurrence of possible modifications in the natural structure of local communities, and also reduce the local biodiversity in freshwater bodies close to or under the influence of these sites (CISZEWSKI et al. 2013; DURÁN; RAUCH; GASTON, 2013; HOLOPAINEN et al. 2003; LEFCORT; VANCURA; LIDER, 2010).

In this context, the first uranium mine to be explored in Brazil (Uranium Osamu Utsumi Mine) is located in the Poços de Caldas plateau region (Minas Gerais, Brazil), where the activities started in 1982, with a total production of 1.030t U by 1995 (CIPRIANI, 2002). This mining area is located in the dependencies of the Mineral Treatment Unit of the Brazilian Nuclear Industries (UTM/INB), and since it is situated in a region characterized by presenting the anomaly of elevated natural radioactivity associated with volcanic rock and mineral uranium deposits, it has aroused the interest of researchers, originating characterization studies mainly in the fields of Physics, Chemistry, Geology, Radiology and Biology.

At the UTM/INB site, low uranium ore tailings in the sterile piles associated with iron pyrite sulfide (FeS_2), in the presence of water and oxygen, provide adequate conditions for the occurrence of AMD, leading to the formation of radioactive acid effluents. The occurrence of AMD is very common in uranium mining areas, since the majority of the U is associated with pyrite, which, under favorable environmental conditions (principally exposure to air and water) forms H_2SO_4 and $FeSO_4$ and then $Fe(OH)_3$, the latter being responsible for the red or orange color indicating the occurrence of AMD (MKANDAWIRE, 2013).

The radioactive effluents continuously produced at UTM/INB present relevant concentrations of the following chemical species: fluoride, sulfate, manganese, zinc, uranium, aluminum and others (NASCIMENTO; FUKUMA; HORTELLANI, 1998), constituting a complex metal mixture. The chemical *in natura* UTM/INB effluent treatment involves the use of calcium hydroxide, calcium oxide and barium chloride to raise the pH value, providing conditions to precipitate the stable and radioactive metals (NÓBREGA; LIMA; LEITE, 2008). After the chemical treatment, the stable and radioactive metals are precipitated in decantation basins, and the supernatant subsequently discharged into water bodies in the region, that is, into the Antas reservoir.

The UTM/INB site is currently in the closing down phase, but Brazil does not have the technology or systematized experience for the remediation and decommissioning of uranium mines and plants. Hence the regulatory organs, both nuclear and environmental, have the legal responsibility to analyze their safety so as to determine the long and short term consequence

to humans and to the environment. With respect to protection of the environment, the current approach for the radiological protection of non-human species foresees determination of the effects of metal and radionuclide dose and concentration rates on plants and animals (ICRP-91, 2003; ICRP-108, 2008). Considering this focus, one applied analytical tool could be the eco-toxicity tests, which are used to detect and evaluate the inherent capacity of the toxic agent to cause deleterious effects on live organisms.

Thus the results of the ecotoxicological tests and chemical analyses generated in the present study could contribute to a better long and short term evaluation and forecast of possible impacts of uranium mining activities on the aquatic biota of tropical aquatic ecosystems, including the Antas reservoir. This is a proposal of an applied nature, making it possible to apply the results to other situations where contamination by stable and radioactive metals resulting from uranium mining activities has occurred. Hence with a view to contributing to knowledge concerning the above, the general objectives of the present study were to evaluate the water quality of samples of *in natura* and treated effluent originating from uranium mining with acid mine drainage (UTM/INB) by way of an integrated chemical and ecotoxicological approach, applying the same approach to water samples taken from the reservoir under the influence of this mining company.

1.2 Ecotoxicology and the Cladocerans

The toxicity tests are based on the fundamental principal that the identification of the response of live organisms to exposure to toxic agents is dependent on the quantity (exposure level) of these agents (HOFFMAN et al., 1995). According to Cooney (1995), the quantification of toxic stress on the aquatic biota depends mainly on methods based on acute and chronic tests. Such tests have the objective of evaluating the inherent effects of one or more substances and/or elements of interest (for example: environmental samples and/or metals) on the test species in a given time period (GHERARDI-GOLDSTEIN, 1988). Acute toxicity tests are generally defined by their short duration period (usually 2 to 4 days), and are carried out to quantify the effect of toxic agents that lead to immobility and lethality to the organisms during a short period in their life cycle. On the other hand, in chronic toxicity trials the more frequently observed evaluation criteria are concerned with reproduction (embryo, larva, juvenile and adult), covering the more sensitive life phases of the organisms (ABNT, 2009; ABNT, 2010; APHA, 1995).

Toxicity trials with aquatic organisms should be carried out with selected taxonomic groups considered representative of the aquatic ecosystems. Standardization of the use of test species is recommended, since the requisites for their use in toxicity tests are well known and established. In this context, the daphnids stand out amongst the invertebrates of sweet waters most frequently used as test organisms, since they are widely used in acute and chronic ecotoxicological evaluations (ABNT, 2009; ABNT, 2010; ASTM, 1992c; e; i). The daphnids are small freshwater micro-crustaceans commonly known as "water fleas", belonging to the Class of Crustacea, Order of Cladocera and Family of Daphniidae, which includes the *Daphnia* spp. (*D. pulex*, *D. magna*) and the *Ceriodaphnia* spp. (*Ceriodaphnia dubia*), ubiquitous in temperate freshwaters (BERNER, 1986). On the other hand the freshwater species *Ceriodaphnia silvestrii*, ubiquitous in tropical regions, is also widely used as a test organism in toxicity trials (ABNT, 2010; FONSECA; ROCHA, 2004). In freshwater habitats the daphnids *Daphnia* spp. and *Ceriodaphnia* spp. are considered ecologically relevant, since they convert the phytoplankton, the bacteria and other suspended particles into animal protein, as well as contributing with a significant part of the diet of many fish (COONEY, 1995).

The use of daphnids in ecological tests is related to some important intrinsic characteristics of these organisms, such as a relatively short life cycle, easy to cultivate and handle in the laboratory, parthenogenetic reproduction with the production of clones, wide distribution in freshwater bodies, and sensitivity to a wide range of aquatic contaminants (COONEY, 1995; LILIUS; HÄSTBACKA; ISOMAA, 1995).

The cladoceran undoubtedly most used in toxicity trials of ecotoxicological tests is the species *Daphnia magna* (ADEMA, 1978) due principally to the large size of the newborn, making them easy to observe. On the other hand the distribution of this species is restricted to temperate environments with high and medium northern latitudes, where the natural waters contain large amounts of carbonate and are therefore characterized as hard (MITCHELL; HALVES; LAMPERT, 2004; TERRA; FEIDEN, 2003). The majority of ecotoxicological studies in freshwater bodies are carried out in temperate regions, and therefore it is natural that the majority of the standard organisms used belong to these regions, such as *Daphnia magna*, since regions from tropical and arctic regions are rare amongst the recommended standard organisms (NIKINMAA, 2014). However the use of native species in tropical regions such as Brazil has recently been strongly recommended (ABNT, 2009; 2010; FONSECA; ROCHA, 2004; FREITAS; ROCHA, 2010), since such species are considered ecologically more relevant, reflecting local conditions more closely. In addition the use of indigenous species can explain differences in water quality, such as soft water, characteristic

of tropical regions (HARMON; SPECHT; CHANDLER, 2003), where *D. magna* is considered exotic.

1.3 Uranium mining activities and AMD

Uranium mining has the potential to liberate radionuclides in addition to other stable metals in aquatic bodies close to the area, causing changes in the chemical and biological characteristics of the environment (ANTUNES et al., 2007b). According to Elbaz-Poulichet et al. (1999), uranium mining activities, as also other processes associated with this activity, can lead to metal and uranium enrichment in waters and rivers downstream from the sites, mainly due to inappropriate discharge (e.g. discharge of radioactive effluent into aquatic bodies). AMD frequently occurs in uranium mines due to the presence of metal sulfides, generally pyrite (FeS_2), associated with rocky matrixes, which, under favorable environmental conditions, promote AMD, which is related to the production of elevated amounts of radioactive effluent. It should be emphasized that the radionuclides can enter the biota present in the fresh or salty water systems by absorption from the water, sediment and food. The radionuclides tend to accumulate at the bottom of sediments, but can enter the organic debris of the reservoirs after the death of plants and animals, which can sequester the radionuclides in their tissues when still alive. Thus the resuspension or dissolution of the debris allows the radionuclides to be remobilized to other areas within the system, or even enter the food chain (COONEY, 1995). With respect to the toxicity of the metals, including the radioactive metal uranium, it should be mentioned that this can vary according to the aquatic species used, due to the intrinsic characteristics of each one and/or different genotypes of the same species (BAIRD et al., 1991), as also the environmental conditions related to water quality (e.g. hardness, pH value, alkalinity, dissolved organic matter), which tend to greatly affect chemical speciation and hence the bioavailability of the metals in the environment in question.

Different international organizations have recognized the need to guarantee that man, as also the environment at its different organizational levels, be adequately protected against the effects of radioactive substances in environments impacted by nuclear installations (IAEA, 2006; ICRP-91, 2003; ICRP-108, 2008; OECD-NEA, 2007). In 2003, the “International Commission on Radiological Protection (ICRP)” published a document concerning the importance of the environment, considering the impacts of ionizing radiation. Thus studies are required to determine the safe dose and/or concentration rates of radionuclides for the

maintenance of the biota in order to attend the interests of the radiological protection of non-human species (ICRP-108, 2008). This focus, aimed at protecting the environment from the noxious effects of ionizing radiation, finds support in the application of ecotoxicology, a science which studies the effects of different toxic agents on the organisms present in the ecosystems.

In this context, studies of aquatic systems located in uranium mining regions in Australia (RIPON; RILEY, 1996), in Portugal (ANTUNES; PEREIRA; GONÇALVES, 2007a), in Canada (PYLE; SWANSON; LEHMKUHL, 2001; 2002; ROBERTSON; LIBER, 2007) and in The Czech Republic (HUDCOVÁ; BADUROVA; ROZKOSNY, 2013) showed evidence that the results of ecotoxicological analyses complemented the physical, chemical and radiological data, contributing to a better evaluation and forecast of the risks caused by discharging uranium mining debris into the environment. According to Sheppard et al. (2005), an evaluation of the risk of the impact caused by the majority of radionuclides is based on the dose rate received by the organism of interest. In addition, according to these authors, in the case of uranium, the risk coming from the chemical toxicity is more relevant than the risk of radiological toxicity. Thus the radioactive metal uranium is considered to be one of the greatest concerns in water bodies under the influence of uranium mines, since it is considered potentially toxic for freshwater biota (ANTUNES et al., 2007). According to the literature, studies have related the toxic and acute effects of this metal to freshwater invertebrates such as: the cnidarian *Hydra viridissima* (RIETHMULLER et al., 2001), the alga *Chorella vulgaris* (CHARLES et al., 2002; FRANKLIN et al., 2000), the macrophyte *Ceratophyllum demersum* (MARKICH, 2013), the worm *Tubifex tubifex* (LAGAUZÉRE; TERRAIL; BONZOM, 2009), the mosquito *Chironomus tetans* (MUSCATELLO; LIBER, 2009) and the cladoceran *Daphnia magna* (MASSARIN et al., 2010; POSTON; HANF; SIMMONS, 1984; ZEMAN et al., 2008).

In Brazil, in studies developed at the Antas reservoir (located in the hydrographic sub-basin of the Ribeirão das Antas river), which is under the influence of a uranium mine (UTM/INB), Ronqui et al. (2010) related the low density of planktonic organisms in this reservoir to the discharge of treated effluent coming from the UTM/INB demonstrating the effect of radioactive effluent on the biota. It should be pointed out that in natural environments, although organisms can help in detecting the impacts, it is difficult to establish a cause and effect relationship for the qualitative and quantitative population changes that occur in these ecosystems. Thus the majority of the quality standards used to protect aquatic

life were, and still are, established based on laboratory studies which simulate what can occur in aquatic ecosystems due to the introduction of toxic agents reasonably close (BERTOLETTI, ZAGATTO; 2006). In other studies carried out at the Antas reservoir by Azevedo et al. (2010), Ferrari et al. (2010) and Rodgher et al. (2013), concentrations of the chemical species (uranium, manganese, fluoride, zinc and aluminum) above the limits established by current legislation (CONAMA 357/2005) were detected. It should be emphasized that the values for U determined in samples of water taken from the hydrographic sub-basin of the Ribeirão das Antas river in the above-cited studies were above the limit considered safe (concentration without toxic effect equal to 0.005 mg L^{-1} U) for freshwater invertebrates, as proposed in a review by Sheppard et al. (2005). In another study also carried out in the hydrographic sub-basin of the Ribeirão das Antas river, the results registered that water samples with a concentration of 0.062 mg L^{-1} U) caused chronic toxicity in the species *Ceriodaphnia dubia* (ARCAL RLA01/10-2009 project).

1.4 Justification

In this context, the need to constantly maintain and amplify ecotoxicological studies allied with the chemical characterization of water systems influenced by nuclear installations, should be emphasized, aiming for a better evaluation and forecast of the long and short term impacts of these installations on the environment. In addition, further information concerning the toxicity of effluents or of natural waters contaminated with uranium and other metals on the aquatic biota in tropical environments is scarce. Thus additional studies are absolutely necessary to contribute to the determination of safe predicted concentrations of toxic metals such as uranium, for aquatic organisms.

This is a proposal of an applied nature, which would make it possible to use the results of the present study in other situations where contamination by metals caused by uranium mining activities occurs.

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2 OBJECTIVE

2.1 General Objective

The objective of the present study was to evaluate the water quality of *in natura* and treated effluent samples coming from a uranium mining site with acid mine drainage (UTM/INB), and of water samples taken from a tropical reservoir under the influence of this mine, using an integrated chemical and ecotoxicological approach. With a view to complementing this ample approach, acute toxicity tests with the metals of interest uranium and manganese were also carried out, and the chemical speciation of the uranium and its relationship to toxicity to daphnids determined.

2.2 Specific objectives

- ✓- Determine the sensitivity range of the species *Ceriodaphnia silvestrii* to the reference substance sodium chloride and of *Daphnia magna* to potassium dichromate under the cultivation conditions established in the Ecotoxicology Laboratory of LAPOC/CNEN, for use in the toxicity tests;

- ✓- Seasonally evaluate the chemical quality and acute toxicity potential of *in natura* and treated effluent samples from the UTM/INB, and also water samples from the Antas reservoir.

- ✓- Determine the acute toxicity (CE_{50}) of the metals manganese and uranium for *C. silvestrii* and *D. magna* using standard reference substances so as to evaluate the toxic potential of these metals as compared to the values registered in water samples taken from the Antas reservoir;

- ✓- Determine the speciation of the uranium at pH 7 and pH 8 for the species *D. magna* and at pH 7.5 for the species *C. silvestrii*;

- ✓- Evaluate the efficiency of the system used to treat the *in natura* effluent from UTM/INB by way of a chemical characterization and acute toxicity tests with daphnids;

- ✓- Determine the acute toxicity potential (CE_{50}) of the *in natura* effluent from UTM/INB.

3 MATERIALS AND METHODS/RESULTS AND DISCUSSION

CHAPTER 3.1 Effects of the discharge of uranium mining effluents on water quality in the reservoir: a limnological and ecotoxicological integrative assessment

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Abstract. The water quality in Antas reservoir, under influence of treated effluents from a uranium mining area Ore Treatment Unit (UTM) with acid mine drainage, was investigated. Samples were collected in the Antas reservoir (CAB, P41-E and P14) and in the UTM (P41-S), quarterly. Limnological and acute 48 h toxicity tests using *Ceriodaphnia silvestrii* and *Daphnia magna* analyses were carried out to determine the potential environmental risks due to the discharge of uranium mine effluents into this reservoir. For all the water samples taken from the treated effluent (P41-S) were positively correlated with elevated concentrations of uranium, manganese, aluminum, zinc and fluoride and with high electrical conductivity and pH values, being considered toxic. Water samples taken from the reservoir showed chemical concentrations above the legislation limits: in November 2014 for fluoride (4.5 mg L^{-1}) uranium (0.082 mg L^{-1}), sulfate (662.4 mg L^{-1}), manganese (1.125 mg L^{-1}) and aluminum (1.55 mg L^{-1}), and in July 2015, for fluoride (2.55 mg L^{-1}), uranium (0.01 mg L^{-1}) and manganese (0.36 mg L^{-1}); the extremely high average value for hardness (543.55 mg L^{-1}) possibly reduced the toxicity potential of this chemical species mixture with respect to the bioindicators. The influence of the variation in water hardness on the toxicity of the cladocerans was discussed.

3.1.1 Introduction

Historically mine sites are a major source of contamination to aquatic environments. Countries worldwide face a severe environmental problem due to deactivated uranium mines. Environmental surveys carried out around U mining sites are generally based on physicochemical and dosimetric measurements, without taking biological effects into account[1,2]. The simultaneous presence of a large number of radioactive and stable chemical species in uranium mine effluents increases the challenge of assessing the toxicity of such complex mixtures, whose effects on the environment are still to be fully understood[3]. Ecotoxicological research and the effects of these chemicals on the aquatic ecosystems surrounding mining areas has mainly focused on temperate countries, while little information is available about tropical ecosystems. The few studies carried out in aquatic systems situated in uranium mining regions including ecotoxicological analyses and physical and chemical parameters, were carried out in Australia[4], Portugal[3], Canada[5,6], the United States of America[7] and the Czech Republic[8], indicating the need to expand the knowledge concerning the ecotoxicological approach to these particular situations outside these countries.

In Brazil (Caldas, Minas Gerais State) there is a uranium ore-mining area (Ore Treatment Unit of the Brazilian Nuclear Industries - UTM/INB), the main environmental problem of which is the generation of acid mine drainage (AMD) containing high concentrations of fluoride, sulfate, manganese, zinc, aluminum and uranium. Before being discharged into the environment (Antas reservoir), this uranium mine effluent is treated with slaked lime, contributing to the high hardness values registered in water samples from the Antas reservoir [9,10,11,12]. AMD is frequently observed in mine sites that contain sulfide rocks, being caused by the oxidation of metal sulfides (mainly pyrite) to produce sulfuric acid and discharge metals of potential toxicity[13,14]. This represents an important source of water quality degradation throughout the world[15], since its continuous release into the environment is related to a severe pollution problem associated with these mining activities. Thus much attention has been paid to the degradation of aquatic ecosystems downstream from mine sites affected by AMD[15,16,17,18].

The aquatic environment usually represents the final destination of contaminants from problematic areas, where they can affect the local biota[19]. In studies about the assessment of environmental contamination, the integrated approach between physicochemical analyses and toxicity tests is considered an efficient strategy to better comprehend the ecological effects of releasing treated effluents into the freshwater system[20,21]. Recently, authors have suggested

that an ecotoxicological characterization of the aquatic environment under the influence of the UTM/INB should be carried out, in order to better assess the risk of toxic effects and consequences of the chemical species in this particular case, with respect to the aquatic biota[11,12]. According to Goulet et al.[22], more toxicity studies with water chemistry downstream from U mines and mills are necessary, to support predictive assessment of the impacts of U discharge into the environment.

In this context, the present study was designed to evaluate the relation between the limnochemical and ecotoxicological approaches, taking seasonal and spatial samples from the reservoir under the influence of treated effluents coming from the uranium mine and containing AMD. Acute 48 h toxicity tests were carried out with *Ceriodaphnia silvestrii* and *Daphnia magna*, to assess possible risks caused by the uranium mine effluents discharged into the tropical freshwater systems. Moreover this represents a proposal of an applied nature, allowing one to fill in gaps in current knowledge at other sites where freshwater contamination occurs due to the presence of mixtures of stable and radioactive metals proceeding from uranium mines.

3.1.2 Materials and methods

3.1.2.1 Description of the study site and sampling

The UTM/INB was the first uranium deposit to be exploited in Brazil and is located in the Poços de Caldas Plateau (Minas Gerais State, Brazil) at 1291 m above sea level. Uranium production in this mining area started in 1982 and lasted for 13 years, generating a total of 1242 tons of U_3O_8[23]. The open pit mine covers an area of about 2 km^2. In the development of the mine, 44.8 10^6 m^3 of rock were removed, and of this amount, 10 M tons were used as building material (roads, ponds, etc.), the rest being disposed of in two major waste rock piles. The milling process generated approximately 2.39 10^6 m^3 of tailings that were disposed of in a dam with an area of 0.86 km^2. Mining activities no longer take place, but the chemical plant in charge of the liquid effluent treatment is still active[24,25]. The acid effluent is treated by chemical processes with calcium hydroxide and oxide (slaked lime) and flocculating agents, and then directed into a decantation trough for sedimentation of the stable and radioactive metals. After treatment, the effluent is released into the Antas reservoir. The Antas reservoir is located in the southeast of Minas Gerais State and has a volume of 3.9 x 10^6 m^2 and a drainage area of 51 km^2 with an average depth of 4 m (maximum of 8 m).

Climatically, the region is classified as Cwb according to the Köppen criteria, between group A (tropical and hot) and group C (mesothermic conditions - dry winter and rainy summer). The minimum and the maximum temperatures vary between 12 °C and 25 °C. The average annual rainfall is around 1,700 mm[26].

The present study involved the evaluation of three sampling stations within the Antas reservoir (CAB, P41-E and P14) and one point in the area of the UTM/INB (P41-S). The selected sampling sites were located upstream and downstream of the treated effluent discharge: P41-S corresponded to treated effluent before being discharged into the Antas reservoir; CAB was upstream of the mining effluent discharge, located at the head of the reservoir and about 1.5 m deep; P41-E was shallower 1.0 m deep and near the middle of the reservoir, where the treated effluent from the UTM/INB was discharged; and P14 was downstream of P41-E, near the reservoir dam, and deeper than the other sites, about 6.9 m deep[11,27] (Figure 3.1.1). Sampling was carried out during four periods: November 2014, February, July and October 2015. The water samples were collected using 5-L Van Dorn bottles. The limnochemical analyses and acute 48 h toxicity tests were carried out on all the water samples from the Antas reservoir and on the treated effluent.

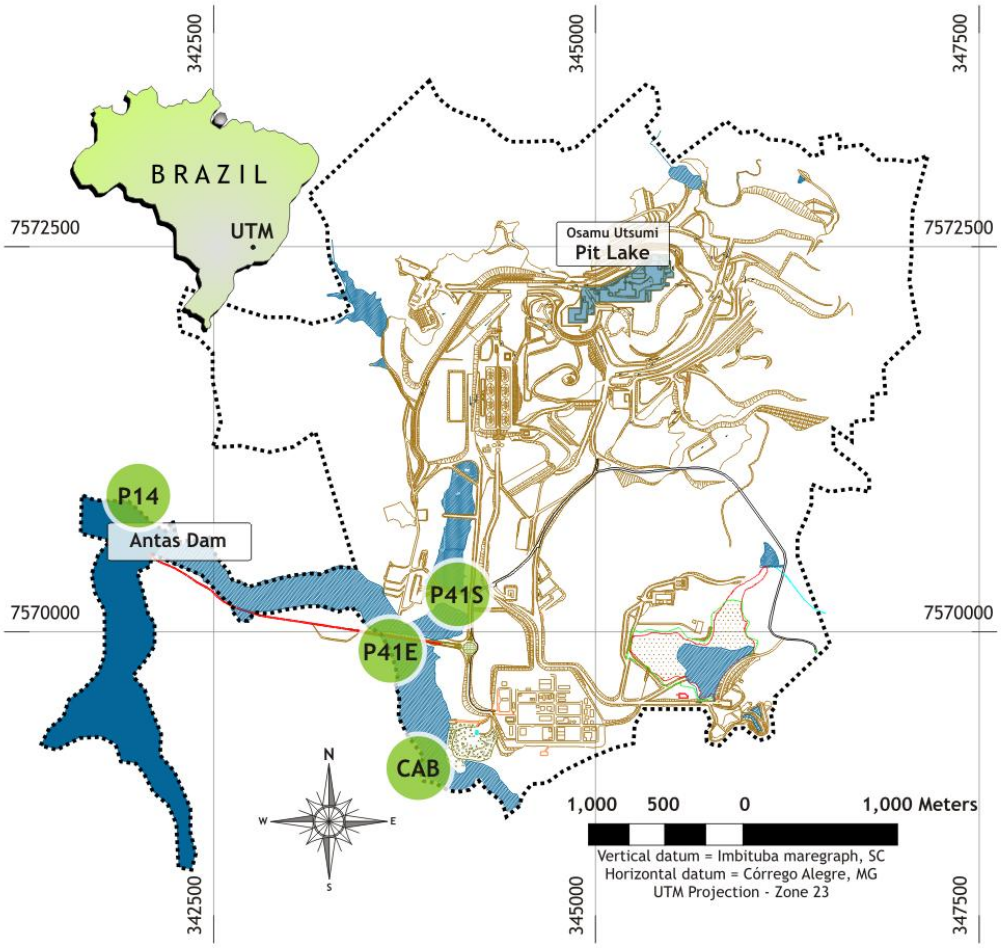

Figure 3.1.1 Location of the sampling points in the Ore Treatment Unit/ Brazilian Nuclear Industries (UTM/INB) and in the Antas reservoir. Figure 1 was produced in ArcGIS software version 9.3.1, by Alberti H.L.C and Filho E.O.L).

3.1.2.2 Limnochemical analyses

Water electrical conductivity, dissolved oxygen, pH and temperature were measured using a Horiba®, model U-10 multiparameter probe field meter. The reduction-oxidation potential was determined using a Digimed® model DM-22 Pt electrode (with Ag/AgCl as the reference). Water samples were taken to determine the chlorophyll *a* concentration according to Lorenzen[28], and the total suspended solids were analyzed according to Teixeira et al.[29]. The total nitrogen and total phosphorus contents were also determined[30].

The hardness of the water (Ca^{2+} and Mg^{2+}) and the metals were quantified by inductively-coupled plasma atomic-absorption optical emission spectrometry (Varian®, model Liberty RL). Fluoride was estimated potentiometrically with an ion-selective electrode and

sulfate by UV-Vis spectrophotometry (Varian®, model Cary 50)[31]. The water samples taken for metal determinations were preserved by adding nitric acid to pH < 2 (Merck®, Ultrapure acid) and storing at 4 ^{o}C. Total metal concentrations (aluminum, total iron, barium, manganese and zinc) were determined according to U.S. EPA[32], method 6010C and measured by atomic emission spectrometry (Varian®, model Libert RL). The uranium concentrations were measured using induced coupled plasma mass spectrophotometry (ICP-MS, Perkin Elmer, model NexIOn 300) following methods 3030E and 3125B according to APHA[30].

The chemical and physical data obtained for the water sampled from the Antas Reservoir and treated effluent were compared with the limits adopted by the Brazilian guidelines: Conama Resolution 357 Class II, Conama Resolution 430 and Ofício Cnen SLC/50[33,34,35].

3.1.2.3 Ecotoxicological analyses

The *C. silvestrii* cultures were kept under a controlled temperature (24 ± 2 °C) and photoperiod (16:8 h light/dark) in reconstituted water with pH values between 7.0 - 7.6, electrical conductivity of 160 µS cm^{-1} and hardness between 40 and 48 mg L^{-1} $CaCO_3$ [36] (Appendixes A) The *D. magna* cultures were kept in M4 culture medium with hardness between 175 and 225 mg L^{-1} $CaCO_3$, pH 7.0 to 8.0, electrical conductivity of 160 µS cm^{-1}, temperature of (20 ± 2°C) and photoperiod of (16:8 h light/dark)[37,38] (Appendixes B).

Stock cultures of the green alga *Raphidocelis subcapitata* were maintained in CHU culture medium[39] (Appendixes C). The algae were centrifuged and resuspended in the appropriate medium for *C. silvestrii* and *D. magna*. Both cultured aquatic animals were fed daily on *R. subcapitata* chlorophycean algae (Appendixes D). For *C. silvestrii*, the concentration of the algal suspension ranged from 1 to 5 x 10^5 cells mL^{-1} per organism, while for *D. magna*, the concentration provided at each renewal was of 1 x 10^6 cells mL^{-1} per adult organism. In addition, the fish food supplement Tetramin®, diluted in processed water (Millipore®, model MilliQ gradient) to a concentration of 5.10^3 mg L^{-1}, was employed in 40 mL aliquots. Each aliquot was added weekly to a suspension of dry Fleishmann® yeast at a concentration of 0.2 g per 40 mL of processed water, and stored at 4 °C.

According to international and national norms, the 48 h acute toxicity tests with the reference substances $K_2Cr_2O_7$ for *Daphnia magna*[38,40] and NaCl for *Ceriodaphnia silvestrii*[36], were applied in order to assure that the test conditions were reliable. According to the results of the sensibility tests carried out, the substance NaCl presented an effective inhibitory

concentration (48 h EC_{50}) for *C. silvestrii* at average values of 1.33 mg L^{-1}. With respect to *Daphnia magna*, average values of 1.08 mg L^{-1} of the substance $K_2Cr_2O_7$ provided an effective inhibitory concentration (48 h EC_{50}). These values for *C. silvestrii* and *D. magna* were within the prescribed ranges set by the guidelines.

The 48 h acute toxicity tests using *C. silvestrii*[36] and *D. magna*[38,40] as the test organisms were carried out with each water sample from the Antas reservoir (CAB, P41-E and P14) and with the samples of treated effluent (P41-S) before being discharged into the Antas reservoir. In the tests, animals that were less than 24 hours old were divided into four groups (replicates) of five animals each, and exposed to 10 mL (*C. silvestrii*) and 50 mL (*D. magna*) of each sample in polycarbonate beakers (Brand®). For all the bioassays, control treatments were prepared with dilution water. The experimental conditions (temperature and photoperiod) and dilution waters used in these experiments were the same as those used to culture the organisms. The pH values (Micronal®, model B374 potentiometer), electrical conductivity (Orion®, model 145A conductivimeter) and dissolved oxygen (WTW®, model OXI 316i environmental oximeter) of the effluents and water samples tested were determined at the beginning and end of all the acute toxicity tests.

In addition to the 48 h acute toxicity tests carried out with the environmental samples, laboratorial 48 h acute toxicity tests with synthetic media, exposing the daphnids (*C. silvestrii* and *D. magna*) to the following hardness values: 152, 276, 350, 709 and 872 mg L^{-1} as $CaCO_3$, were also carried out. The aim was to verify the survival potential of these bio-indicators in the high hardness values registered for the environmental samples used in the present study. The laboratorial tests were carried out with both daphnids in the modified dilution water according to ISO 6341:2012[38]: $CaSO_4.2H_2O$ (1500 mg L^{-1}); KCl (200 mg L^{-1}); $NaHCO_3$ (4800 mg L^{-1}); $MgSO_4 .7H_2O$ (6100 mg L^{-1}), presenting a hardness value of 44 mg L^{-1} $CaCO_3$. The higher hardness values (152 to 872 mg L^{-1} as $CaCO_3$) were obtained by appropriate dosing of the dilution water with $CaSO_4.2H_2O$.

3.1.2.4 Statistical analysis

The results of the physical and chemical analyses were analyzed using ANOVA and Tukey's test (post hoc test) to detect significant differences between the water samples obtained from the different sites at the Antas reservoir at the different collection times. The above statistical tests were carried out using the BioEstat 4.0 program[41]. Fisher's exact test was used to distinguish significant differences in the survival of the cladocerans between the

control treatment and the Antas reservoir and treated effluent water samples. The multivariate analysis was then used to extract the main characteristics from this large data set, and PCA was used to extract and identify the principal sources of variation and the relationship of the variables and samples to these, and hence reduce the number of data without losing information[42].

3.1.3 Results

3.1.3.1 Limnochemical analyses

Table 3.1.1 shows the variation in the physical and chemical parameters of the water samples from the treated uranium mine effluent and the Antas reservoir. The average concentrations of the ions determined in the water samples from the treated effluent (P41-S) were as follows: aluminum (0.58 mg L^{-1} ± 0.22), fluoride (15.21 mg L^{-1} ± 9.96), total iron (0.62 mg L^{-1} ± 0.68), manganese (0.80 mg L^{-1} ± 0.52), sulfate (428.51 mg L^{-1} ± 268.59), uranium (0.04 mg L^{-1} ± 0.03) and zinc (0.72 mg L^{-1} ± 1.19). With respect to hardness, the concentrations varied from 543.84 to 1115.15 mg L^{-1}. The average values for electrical conductivity and pH were 1137.76 μS cm^{-1} ± 211.05 and 8.20 ± 0.87, respectively. The values for manganese in November 2015 (1.12 mg L^{-1}) and July 2015 (1.38 mg L^{-1}); fluoride in February 2015 (12.55 mg L^{-1}) and July 2015 (32.06 mg L^{-1}) and for pH in October 2015 (9.49) in the samples from P41-S exceeded the limits defined by the Conama Resolution 430[34].

Table 3.1.1 Minimum and maximum values found for the physical and chemical variables in water samples obtained from the sampling points. Temperature (T), hydrogen potential (pH), dissolved oxygen (DO), electrical conductivity (EC), reduction oxidation potential (Eh), chlorophyll *a* (Chl), total nitrogen (Ntot), total phosphorus (Ptot), Hardness, fluoride (F^-), sulfate (SO_4^{2-}), suspended solids (SS), aluminum (Al), total iron (Fe), calcium (Ca^{2+}), magnesium (Mg^{2+}), manganese (Mn), uranium (U), zinc (Zn).

Variables	P41-S	CONAMA/430 Effluent discharge	CAB	P41-E	P14	CONAMA/357 Class II
T (^oC)	17.6 - 25.3		17.9 - 25.8	17.7 - 25.0	18.4 - 25.2	
pH	7.12 - 9.49	between 5 - 9	5.79 - 8.02	6.19 - 8.56	6.08 - 7.17	between 6 - 9
OD (mg/L)	2.9 - 7.01		3.38 - 6.11	5.75 - 7.01	3.29 - 7.09	> 5.0
EC (µS/cm)	981 - 1500		26 - 673	66 - 958	108 - 547	
Eh (mV)	98 - 253		201 - 326	160 - 362	160 - 344	
Chla (µg/L)	0.08 - 1.37		0.28 - 2.17	0.39 - 4.12	1.54 - 3.10	30
Ntot (µg/L)	460.1 - 714.1		568.4 - 733.3	309.1 - 547.3	378.1 - 761.6	
Ptot (µg/L)	0.75 - 3.15		1.6 - 16.45	1.33 - 15.45	0.70 - 12.45	50
Hardness (mg/L)	543.8-1115.1		6.61 - 424.2	32.5 - 883.1	41.49 - 323.4	
F^- (mg/L)	6.50 - 32.06	10.0	< 0.50 - 1.43	2.21 - 4.50	1.06 - 2.37	1.4
SO_4^{2-} (mg/L)	10.9 - 754.1		3 - 321.9	12.7 - 662.4	12.4 - 253.4	250
SS (g/L)	3.6 - 15.8		1.2 - 17.3	2.2 - 6.8	1.7 - 11.5	
Al (mg/L)	0.37 - 2.1		<0.1 - 0.293	< 0.10 - 1.55	< 0.1 - 0.131	
Fe (mg/L)	0.049 - 1.58		0.247 - 1.508	0.156 - 1.37	0.12 - 0.31	
Ca^{+2} (mg/L)	216.9 - 445.2		1.89 - 168.0	12.28 - 351.8	15.89-128.1	
Mg^{+2} (mg/L)	0.342 - 0.777		0.384 - 0.949	0.385 - 0.95	0.39 - 0.713	
Mn (mg/L)	0.006 - 1.125	1.0	0.302 - 1.125	0.31 - 1.0375	0.303 - 1.21	0.10
U (mg/L)	0.005 - 0.089		<0.0025 - 0.005	<0.0025 - 0.082	<0.0025 - 0.0145	0.02
Zn (mg/L)	0.017 - 2.79	5.0	<0.01 - 0.093	0.02 - 0.049	0.017 - 0.034	0.18

The two axes of the PCA explained 61.8% of the total variability, 42.2% by the first and 19.6% by the second. It was verified that the PCA (Figure 3.1.2) showed a separation of the samples from point P41-S in relation to the sampling points in the Antas reservoir for the physical and chemical variables throughout the whole period of the study. The samples for point P41-S were positively correlated with elevated concentrations of uranium (+0.293), manganese (+0.229), aluminum (+0.330), zinc (+0.137), electrical conductivity (+0.378), hardness (+0.379), fluoride (+0.246), sulfate (+0.312) and pH (+0.251). On the other hand the results for ANOVA obtained for the water samples from the Antas reservoir revealed significant seasonal differences for some chemical and physical variables. The average water temperature during the rainy period (25 °C in November 2014) was higher than that registered in the dry period (18 °C in July 2015) (P<0.05, Tukey's test). The average suspended solids

contents were significantly higher (P<0.05, Tukey's test) in October 2015 (11.87 mg L^{-1}) than the values observed in July 2015 (2.60 mg L^{-1}). The average pH value was significantly higher (P<0.05, Tukey's test) in July 2015 (7.92) than in November 2014 (6.95), February (6.49) and October 2015 (6.26). The water samples showed average pH values of 7.0. The electrical conductivity of the water showed the highest average value in November 2014 (726 μScm^{-1}) and the lowest in February 2015 (67 $\mu S\ cm^{-1}$) (P<0.05, Tukey's test). The highest average value for the reduction-oxidation potential was recorded for water samples in November 2014 (+329 mV), whereas the lowest value was recorded for water samples in July 2015 (+177 mV) (P<0.05, Tukey's test). With respect to the total phosphorus concentrations, the highest average value was found in the samples collected in February 2015 (14.78 µg L^{-1}), whilst the lowest value was found in October 2015 (1.21 µg L^{-1}) (P<0.05, Tukey's test). The average sulfate concentration was significantly higher (P < 0.05, Tukey's test) in November 2014 (412.6 mg L^{-1}) than in February 2015 (17.3mg L^{-1}). With respect to hardness, the lowest average value was found in February (27 mg L^{-1}) and the highest in November 2014 (543 mg L^{-1}) (P<0.05, Tukey's test). The average value for manganese determined in November 2014 (0.92 mg L^{-1}) was significantly higher (P<0.05) than the values quantified in February (0.38 mg L^{-1}), July (0.32 mg L^{-1}) and October 2015 (0.43 mg L^{-1}).

Spatially, the highest fluoride level (2.91 mg L^{-1}) occurred in P41-E and the lowest (0.96 mg L^{-1}) in CAB and the statistical analysis showed that these two values differed significantly (P < 0.05, Tukey's test).

According to the Conama Resolutions 357 Class II, all the manganese concentrations determined in the water from the Antas reservoir were above the established limit (0.1 mg L^{-1}), while in November 2014, the sulfate concentrations at all the sampling stations were above the limit. Uranium exceeded the permitted limit in water samples from P41-E in November 2014. The fluoride levels exceeded the limits in the samples from CAB in November 2014, from P41-E in November 2014, February, July and October 2015 and from P14 in November 2014 and in February and July 2015[33]. In addition, uranium exceeded the permitted limit (0.008 mg L^{-1}) in water samples from P14 in November 2014 and July 2015[35].

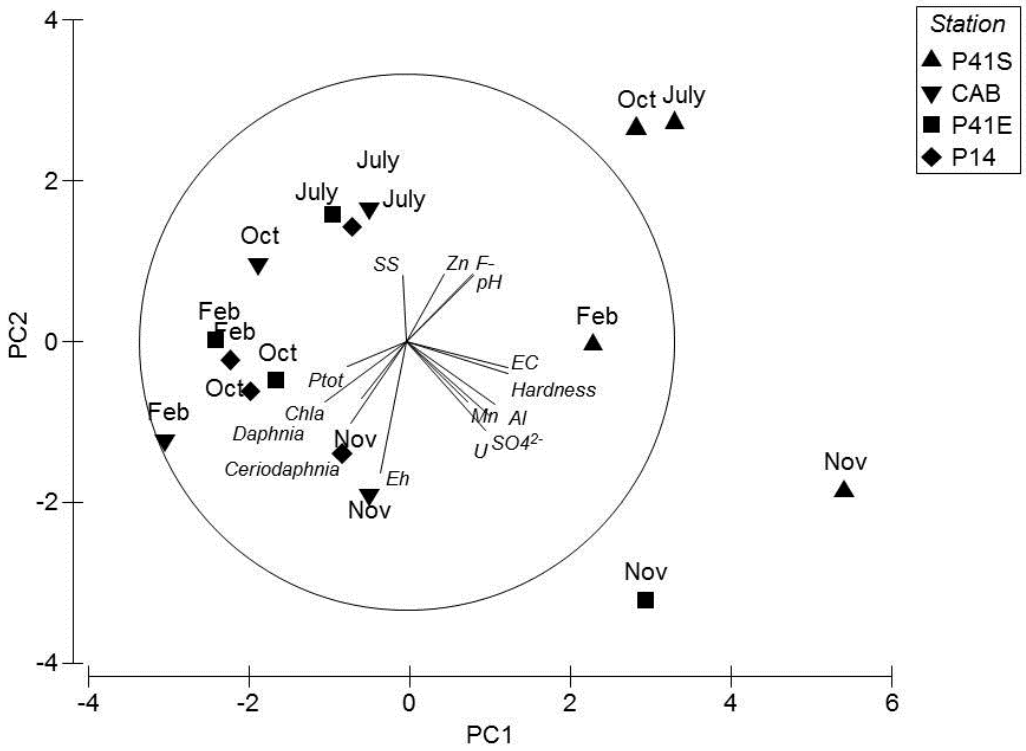

Figure 3.1.2 Principal components analysis (PCA) ordination diagram of sampling points: P41-S (UTM/INB), CAB, P41-E, and P14 (Antas reservoir) in November 2014 (Nov), February (Feb), July (July) and October (Oct) 2015 for variables Ptot = total phosphorus, SS = total suspended solids, EC = electrical conductivity, Hardness, SO_4^{2-} = sulphate, U = uranium, Mn = manganese, Al = aluminum, Eh = reduction oxidation potential, F^- = fluoride, Zn = zinc, pH = hydrogen potential and species *Ceriodaphnia silvestrii* and *Daphnia magna*.

According to the PCA (Figure 3.1.3) the first of the two components explained 59.1% of the total variability, 35.7% for the first axis and 23.4% for the second. It was shown that, in November 2014 and July 2015 the samples from the Antas reservoir showed high positive correlations with the hardness, uranium, electrical conductivity, manganese, fluoride and sulfate values. In addition, in November 2014, the samples from point P41-E showed the strongest positive correlation with the hardness (+0.369), uranium (+0.375), electrical conductivity (+0.324), manganese (+0.350) and sulfate (+0.416) values. On the other hand, in February and October 2015, samples from all the sampling stations were strongly correlated with total phosphorus and suspension solids.

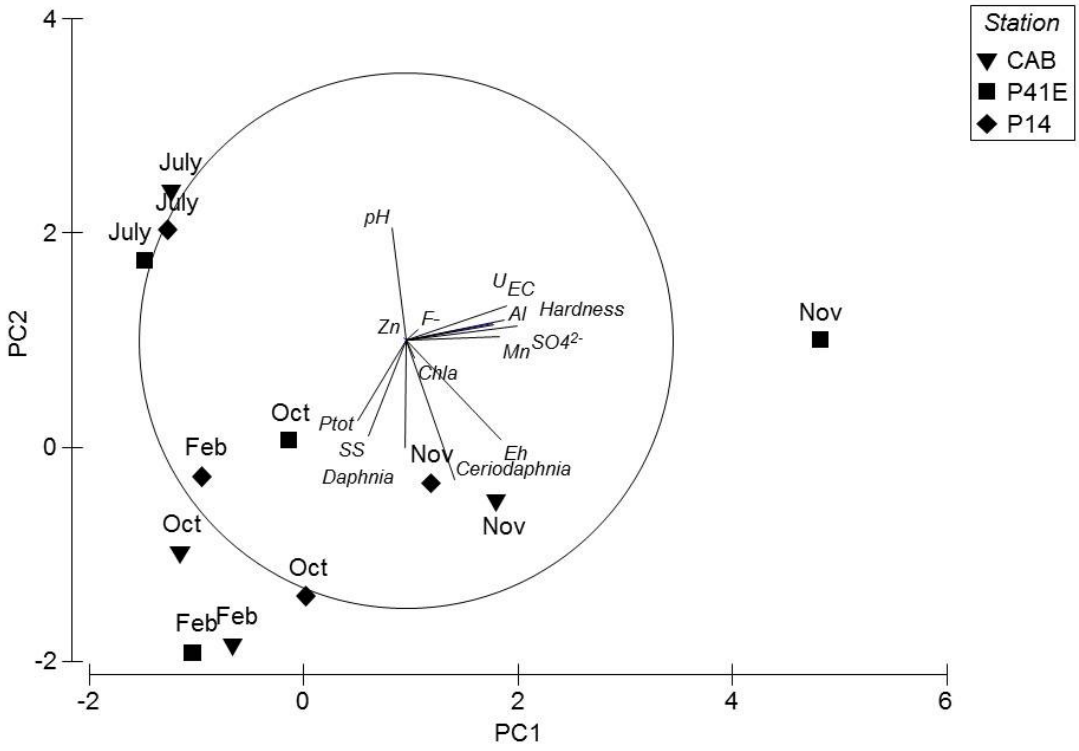

Figure 3.1.3 Principal components analysis (PCA) ordination diagram of sampling points: CAB, P41-E, and P14 (Antas reservoir) in November 2014 (Nov), February (Feb), July (July) and October (Oct) 2015 for variables Ptot = total phosphorus, SS = total suspended solids, EC = electrical conductivity, Hardness, SO_4^{2-} = sulphate, U = uranium, Mn = manganese, Al = aluminum, Eh = reduction oxidation potential, F^- = fluoride, Zn = zinc, pH = hydrogen potential and species *Ceriodaphnia silvestrii* and *Daphnia magna*.

3.1.3.2 Toxicity tests

Figure 3.1.4 (A and B) shows the results obtained in the 48 h acute toxicity tests with the treated uranium mine effluent and water from the Antas reservoir. In the toxicity tests, the survival of the control treatments was 100% for *C. silvestrii* and *D. magna* after 48 h. A statistical analysis of the mobility data from the acute toxicity bioassays with *C. silvestrii* revealed acute toxic effects on the test organisms exposed to water samples from the P41-S (all periods), CAB (July 2015), point P41-E (November 2014, July and October 2015) and P14 (February and July 2015) (Figure 4.1.4A). *Daphnia magna* showed acute toxicity when exposed to water samples from the P41-S (all the periods), CAB (July 2015), P41-E (November 2014) and P14 (July 2015) (Figure 3.1.4B).The results for acute toxicity registered at P41-S showed the effluent did not conform to the standard for discharge into the

environment. The current Brazilian Conama Resolution 430[34] prescribes that the effluent of a polluting source must not cause or present potential to cause toxic effects to aquatic organisms in freshwater bodies.

The toxicity bioassay with *C. silvestrii* indicated an increase in toxicity from CAB to P41-E in November 2014 and October 2015. On the other hand, a reduction in acute toxicity to *C. silvestrii* was detected in the water sample from P41-E in relation to that from point P14 in November 2014 and October 2015. Of a total of 12 samples taken from the Antas reservoir and evaluated for acute toxicity, the frequency of toxic samples was greater for *C. silvestrii* (50%) than for *D. magna* (25%) and all the samples showing acute toxicity for *D. magna* were also toxic for *C. silvestrii.*

According to the PCA (Figure 3.1.3), it was shown that the species *C. silvestrii* presented greater correlation with the water samples from points CAB and P14 in the month of November 2014 with the variables of chlorophyll *a* and Eh. On the other hand the species *D. magna* was more correlated with samples from the points P41-E and P14 in October and February 2015 with the variables of total phosphorus and suspended solids. In July 2015 the tendency of the PCA high correlation with the acute toxicity for the species *C. silvestrii* (-0.521) and *D. magna* (-0.399) in water samples from the majority of the sampling points in the Antas reservoir. However, low correlation with acute toxicity for the species *C. silvestrii* and *D. magna* was indicated for samples from the points CAB in February and October 2015, P41-E in February 2015 and P14 in October 2015, where they presented 100% mobility according to the results obtained in the toxicity assays.

In addition, in the present study the results from the laboratorial experiments showed 100% of survival in the acute toxicity tests for both daphnids when maintained at high hardness values (152 to 872 mg L^{-1} $CaCO_3$).

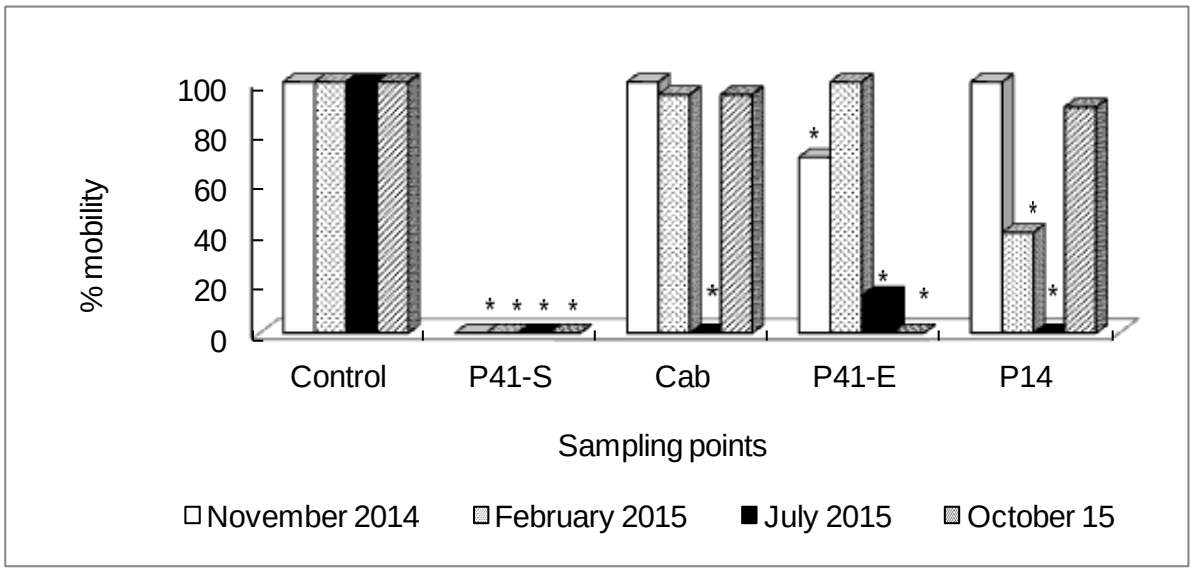

Figure 3.1.4A Results from the *Ceriodaphnia silvetrii* 48 h acute toxicity tests exposed to water samples from sampling points P41-S (UTM/INB) and P41-E, CAB and P14 (Antas reservoir). Figure 4.1.4A. was produced in Microsoft Excel.

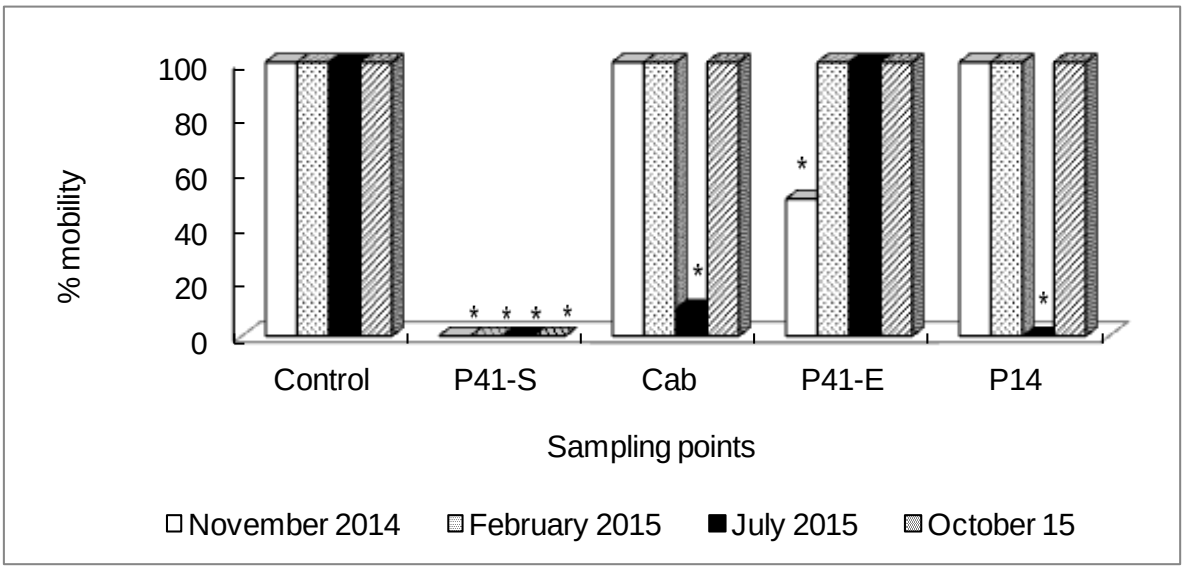

Figure 3.1.4B Results from the *Daphnia magna* 48 h acute toxicity tests exposed to water samples from sampling points P41-S (UTM/INB) and P41-E, CAB and P14 (Antas reservoir). Figure 4.1.4B. was produced in Microsoft Excel.

3.1.4 Discussion

This study provided a comprehensive ecotoxicological and limnological evaluation of a tropical reservoir influenced by uranium mining effluents. In the present research, the average values for sulfate, hardness and manganese were higher in November 2014 than in February 2015. The low values for ions in the water from the Antas reservoir in the wet season (February 2015) can be associated with the high rainfall and low water residence time. Rodgher et al[11] also verified that the Antas reservoir was subject to seasonal variation in relation to the level of chemical compounds. Similar to the present study, Da Silva[43] found high concentrations of metals and sulfate in a stream close to a mining area in Portugal during periods of reduced rainfall. Gemici[44] also found seasonal fluctuations in the physical and chemical water quality data for streams in a mercury mine area in Turkey.

The release of effluents, naturally enriched in metals and radionuclides, is the main legacy of uranium mines. The discharge of uranium mine effluent into the Antas reservoir resulted in contamination with major cations and anions. The uranium concentrations found in the Antas reservoir are equivalent to the concentrations measured in other aquatic ecosystems impacted by U mines, while the Al, Fe and Mn levels were higher[21,45,46]. The results of the physicochemical parameters determined in the water samples from the Antas reservoir also showed that the sulfate, manganese, fluoride, uranium, aluminum[33] and uranium[35] concentrations exceeded the maximum levels according to the Brazilian limits.

Seasonally, according to the present study, in November 2014 and July 2015 the water samples from the Antas reservoir showed high chemical concentrations (F^-, U, and Mn), above the limits established by the Conama Resolution 357, Class II[33] and Ofício Cnen SLC/50[36] and these ion concentrations were also above the values considered toxic for zooplankton species registered in the literature[22,47,48,49,50,51]. In July 2015, a greater frequency of samples toxic for *C. silvestrii* (100%) and *D. magna* (67%) was registered in the Antas reservoir when compared to the results obtained in the other sampling months. To the contrary of that expected, the smallest number of toxic samples was registered for both species in November 2014, that is, only 25%. Based on the principal components analysis (Figure 4.1.3), the values for water hardness probably directed the results obtained in the toxicity tests in November 2014 and July 2015. In November 2014, the extremely high average value for hardness (543.55 mg L^{-1} $CaCO_3$), registered in the water samples from the Antas reservoir, possibly reduced the toxicity potential of this chemical species mixture with respect to the zooplankton bioindicators. On the other hand, in July 2015, when elevated concentrations of

the chemical species were registered and the average value for hardness indicated soft water (59.22 mg L^{-1} $CaCO_3$), acute toxicity was detected for both *C. silvestrii* and *D. magna*. In the literature there is little information related to the influence of water hardness in the expression of metal toxicity in environmental samples. The results for acute toxicity registered in the present study in November 2014 and July 2015 conformed with those of Yim et al.[52], who demonstrated that the toxicity of metal mixtures to daphnids was greatly increased in soft water (44 mg L^{-1} as $CaCO_3$) in relation to the results obtained in a hard water test solution (150 mg L^{-1} as $CaCO_3$). It is known that water hardness affects the toxicity of various metals in many aquatic species[53,54].

Water hardness is a chemical characteristic that depends on the occurrence of alkaline earth metals, mainly calcium and magnesium elements. This feature is due to the dissolution of minerals in soils, and can also be associated with pollution by industrial effluents[55]. It has also been suggested that hard waters, where there are greater Ca^{2+} ion concentrations than Mg^{2+} ion concentrations, offer greater protection to aquatic life with respect to metal toxicity, than aquatic bodies where the greater part of the hardness is due to Mg^{2+} ions[56]. In the present study the hardness water values were related to the treatment of the *in natura* effluent coming from UTM/INB with slaked lime to neutralize the AMD, producing $CaSO_4$. Thus the greater part of the hardness in the Antas reservoir was due to Ca^{2+}, indicating that this element could have acted as a protective factor for the test species in the water samples in November 2014, and could thus have influenced the expression of toxicity.

Major cations may compete with trace metals such as fluoride and sulfate for target cell sites and are known to modify their toxicity for aquatic organisms[57,50]. Kinraide et al.[58] demonstrated that increasing concentrations of cations (e.g. Ca^{2+}, Mg^{2+}) in the exposure medium decreased the negative electrical potential at the cell membrane and hence reduced the electrostatic attraction of the cell membranes for metal ions and decreased the toxicity. On the other hand, Goulet et al.[22] suggested that Ca channels in the cell membranes did not compete with uranium metal for the same uptake sites, instead it appeared that the increase in Ca, Mg and sulfate increased the tolerance of the aquatic organisms to U. Davies and Hall[60] determined that SO_4^{2-} toxicity was inversely related to increasing Ca^{2+} concentrations and Ca:Mg ratios in tests with *D. magna*. Similarly, Shamsollahi et al.[60] found that fluoride ions were less toxic to *D. magna* in hard waters.

Data from the present study clearly showed that the variation in water hardness considerably altered the toxicity of the Antas reservoir samples to the cladocerans during the year. The toxicity bioassay with *C. silvestrii* indicated an increase in acute toxicity from CAB

to P41-E. On the other hand, a reduction in acute toxicity for *C. silvestrii* was detected in water samples from P41-E in relation to the water from point P14. These acute toxicity results agreed with the spatial distribution of the concentrations of uranium, sulfate and fluoride, and with the electrical conductivity values at P41-E (near the site where the mining effluent was discharged), which were higher than those at CAB and P14. In this study the toxicity bioassays indicated that a large proportion of the water samples collected at P41-E and P14 had toxic effects on *C. silvestrii*, possibly resulting from the input of uranium mine treated effluent (P41-S) into the Antas reservoir. The treated effluent samples were positively correlated with elevated concentrations of uranium, manganese, aluminum, zinc and fluoride and with high electrical conductivity and pH values. In addition, the results of the acute toxicity registered at P41-S did not conform to the standards for the discharge of effluent into the environment, according to Conama Resolution 430[34]. The concentrations of chemical species (manganese, fluoride, sulfate and uranium) in the waters at P41-S, P41-E and P14 may represent a risk to invertebrate species.

Thus one must discuss ion toxicity for aquatic invertebrates under different physicochemical conditions, since environmental variables (e.g. water hardness) have an influence on metal and ion toxicity in freshwater systems[61,62]. The environmental variables (e.g. hardness, alkalinity, pH, chloride) which would contribute substantially to modifying the toxicity, can be related to the bioindicator species applied in the toxicity tests (e.g. *Hyalella azteca*, *Ceriodaphnia silvestrii Lemna minor*, *Chironomus dilutes*), the different uptake pathways and the physicochemical composition of the water[22,48,50,51,54,62,63,64].

Freshwater invertebrates in general and daphnids in particular, have been widely used in ecotoxicological studies of freshwater reservoirs contaminated with anthropogenic toxic substances from mining activities. Mejía-Saavedra et al.[49] showed that the river that receives the waters coming from the mining area in Salado, Mexico, were acutely toxic to cladocerans. Chen et al.[17] also demonstrated that the mining effluent made the water of the Pearl River (China) highly toxic to daphnids. Water hardness is a very variable factor, depending on which geochemical region of the world is involved. *Daphnia magna* is a native microcrustacean from Europe, where the natural water contains high carbonate values[66]. Thus *D. magna* was ecologically relevant to this study area because of the high hardness values recorded in the uranium mine effluent and in the water of the Antas reservoir. However, the geographical distribution of *D. magna* is primarily limited to areas of high and middle latitudes[67]. From the viewpoint of ecotoxicity bioassays, it is more adequate to use native species than to import non-indigenous species. In Brazil, the cladoceran *C. silvestrii* is a test

species recommended for ecotoxicological evaluations[36] and in the present study *C. silvestrii* was more sensitive than *D. magna* to acute exposures to the environmental water samples. These results confirm the importance of carrying out acute toxicity tests using an indigenous species such as *C. silvestrii* in the ecotoxicological assessment of tropical aquatic ecosystems around a mining area with high hardness values.

The survival of *C. silvestrii* and *D. magna* was observed in samples from the Antas reservoir with extremely high hardness values (323.4 to 424.2 $CaCO_3$ mg L^{-1} at points P14 and CAB, respectively) in November 2014. In addition, the results of the laboratorial experiments carried out in the present study showed 100% survival in the acute toxicity tests for those cladocerans maintained in high hardness values (152 to 872 mg L^{-1} $CaCO_3$). The survival potential of *C. silvestrii* in very high hardness values, still not registered in the literature, was confirmed by the results of the laboratorial tests carried out in this study. The results showed that extremely high hardness values were not limiting to the survival of the neotropical cladoceran, indicating the suitability of this tropical cladoceran species as a test organism in freshwater with high hardness contents. Acute toxicity of *D. magna* was already reported after exposure to hard water with 600 mg L^{-1} as $CaCO_3$ [69].

3.1.5 Conclusions

This study highlights the usefulness of the ecotoxicological approach associated with the limnological approach as an effective tool to evaluate critical areas within reservoir ecosystems potentially impacted by U discharges to the environment. A clearly protective effect to the acute toxicity of the metal in water from the Antas reservoir was provided to the cladocerans *C. silvestrii* and *D. magna* by very high hardness values. *C. silvestrii* is a native and widely distributed cladoceran throughout South America and was shown to be sensitive to detecting toxic conditions in water samples from the Antas reservoir.

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CHAPTER 3.2 Acute toxicity of manganese for *Ceriodaphnia silvestrii* and *Daphnia magna* in bioassays and the potential toxicity of this metal in uranium mine effluents

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Abstract. Treated effluents coming from the uranium mine (Ore Treatment Unit of the Brazilian Nuclear Industries) containing acid mine drainage (AMD) are continuously discharged into the Antas reservoir, and studies have evidenced that one of the main problems of these effluents when released into the catchment basin of the Ribeirão das Antas river is the high Mn values recorded in the water samples. In the present study acute manganese toxicity effects on *Daphnia magna* and *Ceriodaphnia silvestrii* were determined. The EC_{50} values of both tested species were compared with the Mn values recorded in the environment (Antas reservoir). The Mn 48 h EC_{50} values for *C. silvestrii* and *D. magna* were 5.93 mg L^{-1} and 51.66 mg L^{-1}, respectively ($P<0.05$). According to the literature, the Mn values recorded in the water samples (1.04 to 20.3 mg L^{-1} Mn) from the Antas reservoir revealed a toxicity potential for *C. silvestrii*. Since Mn occurs in the composition of the untreated effluent from UTM/INB, which may contain other stable and radioactive elements, the ecotoxicological monitoring of the water in the Antas reservoir has been suggested since it receives treated effluents coming from UTM/INB, aiming to assess the potential synergistic and antagonistic effects of the chemical mixture that makes up the radioactive effluents that are discharged into this reservoir.

Keywords: Manganese, potential toxicity, uranium mine, effluents, *Daphnia magna*, *Ceriodaphnia silvestrii*

3.2.1 Introduction

The input of metals into the aquatic environment continues, constituting a potential threat for the natural ecosystems, due their direct toxic action on aquatic organisms. Furthermore, many metals can be bioaccumulated and/or biomagnified in food chains, constituting a risk for the predators of the top of the food chain, including man (Loez et al. 1995).

Human concern about metals has mainly focused on non-essential heavy metals (i.e., Pb, Hg and Cd). Manganese is a ubiquitous element considered to be the 12th most common metal and the fourth most abundant, being universally distributed throughout the earth's crust and waters (Siegel and Siegel 2000). In natural freshwater systems the Mn concentration ranges from 1 to 200 μg L^{-1} (Barceloux 1999) and rarely exceeds a concentration of 1 mg L^{-1} (Reimer 1999). Manganese is considered to be an essential element to plants and animals, carrying out an important role in enzyme activation (Rainbow et al. 2002; WHO 2004). Thus since it is considered to be an essential metal its toxicity potential risk has been neglected (Baden and Eriksson 2006). However, pollution by manganese is frequently related to electroplating, metallurgy, the discharge of untreated domestic and industrial sewage, the combustion of combustible fossils, agricultural soil erosion, the formulation and application of pesticides and fertilizers, and also mining activities, mainly when acid mine drainage (AMD) occurs (Shukla and Singhai 1983; U.S. EPA 2004**)**.

AMD is considered to be an important source of water quality degradation, since its continuous release into the environment is related to severe metal pollution due to the large volume of effluents generated, which are known to adversely affect aquatic biota. This acid mixture consists of a large spectrum of chemical pollutants, including the metal manganese. Treated effluents coming from the uranium mine and milling processing (Ore Treatment Unit of the Brazilian Nuclear Industries - UTM/INB) containing AMD, are continuously discharged into the Antas reservoir in Brazil, and studies have shown that one of the main problems of these effluents when released into the catchment basin of the Ribeirão das Antas river is their high Mn values, as recorded in the water samples (1.04 to 20.3 mg L^{-1} Mn) and in the sediment samples (16,000 mg Kg^{-1} Mn) (Campos et al. 2011; Rodgher et al. 2013; Ronqui et al. 2010; Relatório Técnico da Comissão das Águas 2012), often above the current value allowed by Brazilian legislation (Conama 2005). Similarly, manganese pollution of aquatic ecosystems close to mining areas in Australia (Hogan et al. 2005; Van Dam et al. 2008; Harford et al. 2015), Mexico (Gómez-Alvarez et al. 2009) and Portugal (Neves and

Matias 2004; Antunes et al. 2007) have also been detected. As from the growing interaction between the mining-industrial sector and the scientific community, research that provides knowledge and technology to assist the recovery, mitigation and monitoring of the negative impacts caused by the mining sector, will be necessary (Lisieri et al. 2011).

In this context, toxicity tests may be applied as tools to identify the potential toxic effect of uranium mine effluents, and have been widely used as tools to aid in the definition of water quality standards (Bertoletti and Zagatto 2008; Rand et al. 1995). Cladocerans, such as different species of *Daphnia*, are routinely used in toxicity bioassays due to their easy visual examination and short generation time. *Daphnia magna*, although a species whose geographical distribution is restricted to hard-water habitats, is widely used in ecotoxicological bioassays (Lilius et al. 1995). The use of *D. magna* was justified in the present study, because of the high hardness values recorded in the uranium mine effluent and in the water of the Antas reservoir samples. However, the use of indigenous species may reflect a more realistic test of the locality, since these species are representatives of the local flora (Harmon et al. 2003). Thus the use of indigenous species such as *Ceriodaphnia silvestrii*, the species used in the present study, was recommended by the water quality regulators (ABNT 2010). In addition, the use of indigenous species can be related to the generation of more relevant ecological data, reflecting the possible consequences of the presence and/or launching of toxic agents on the main groups of organisms in the environment in question (Freitas and Rocha 2010; Souza et al. 2014).

Since relatively little attention has been directed at the toxicity of Mn for freshwater zooplankton organisms such as species of *Daphnia* and *Ceriodaphnia*, widely used in toxicity tests (Khangarot and Ray 1989; Baird et al. 1991; Boucher and Watzin 1999; Reimer 1999; Lasier et al. 2000), the present study assessed the effects of acute manganese toxicity (48 h immobilization test), using *D. magna* and *C. silvestrii* (tropical species) as the test organisms. The results of the toxicity tests were compared with the values recorded for Mn in the previous year's studies of the treated effluent from the uranium mine and milling processing (UTM/INB) and in the catchment basin influenced by this nuclear installation.

3.2.2 Material and Methods

The *C. silvestrii* cultures were maintained under a photoperiod of (16:8 h light/dark) and controlled temperature (24 ± 2 °C) in reconstituted water with pH values between 7.0 - 7.6, electrical conductivity of 160 µS cm^{-1} and hardness between 40 and 48 mg L^{-1} $CaCO_3$

(ABNT 2010). The *D. magna* cultures were maintained in M4 culture medium with hardness between 175 and 225 mg L^{-1} $CaCO_3$, pH 7.0 - 8.0, electrical conductivity of 160 μS cm^{-1}, temperature of (20 ± 2 °C) and photoperiod of (16:8 h light:dark) (Elendt 1990). Stock cultures of the green algae *Raphidocelis subcapitata* were maintained in the culture medium CHU-12 (Müller 1972).

The cultures of *Ceriodaphnia silvestrii* and *Daphnia magna* were fed on suspensions of the alga *R. subcapitata*. For *C. silvestrii*, an algal suspension concentration ranging from 1 to 5 x 10^5 cells mL^{-1} per organism was provided, while for *D. magna*, the concentration provided at each renewal was of 1 x 10^6 cells mL^{-1} per adult organism (ABNT 2009; 2010).

To carry out the tests, manganese solutions were prepared from a stock solution of $MnCl_2$.4 H_2O (Sigma-Aldrich®) with a targeted nominal concentration of 1000 mg L^{-1} Mn. The water used in the preparation of the test solutions was the same as that employed in the maintenance of the test organisms (reconstituted water). Preliminary tests were carried out according to the Mn concentrations proposed in the literature (Lasier et al. 2000). After the preliminary tests, the stock solution was diluted to produce 7 nominal Mn concentrations for *Ceriodaphnia silvestrii* (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 mg L^{-1}) and 6 for *D. magna* (30, 40, 50, 60, 70 and 80 mg L^{-1}). The highest and lowest nominal manganese concentrations tested were measured at the beginning of the test and after 48 h so as to confirm the nominal Mn concentrations tested, as recommended by ISO:6341 (2012). The manganese concentrations were analytically measured according to U.S. EPA (2007). It should be mentioned that in all the tests with *D. magna*, the medium M4 (Elendt 1990) was not used as the dilution water in the tests with the divalent metal $MnCl_2$. Instead, an alternative medium was used for dilution, as proposed by ISO:6341 (2012).

In the 48 h acute toxicity tests using different manganese concentrations, < 24 h old *C. silvestrii* and *D. magna* neonates were exposed to the Mn concentrations and to the control treatment (only reconstituted water). In the acute tests, 5 individuals were used in 4 replicates, placed in 10 mL polypropylene beakers (Brand®) for *C. silvestrii* and 50 mL ones for *D. magna*, which contained the solution of interest. The tests were carried out in an incubator with a (16:8 h light:dark) photoperiod and controlled temperature of (25 ± 2 °C for *C. silvestrii* and 20 ± 2 °C for *D. magna*), without feeding of the test-organism throughout the 48 h period. The numbers of mobile and immobile organisms in each sample tested were then assessed. Some parameters such as pH, electrical conductivity and dissolved oxygen were measured at the beginning and conclusion of the test. From the test results the mean effective EC_{50} concentrations were determined for both species. As from the nominal metal

concentrations used in each test, the 48 h EC_{50} values of manganese for daphnids were determined using the trimmed Spearman-Karber method (Hamilton et al. 1977).

Since the mean concentrations of the test solutions presented a difference of about 10% in relation to the nominal concentration, the CE_{50} values were reported in terms of the nominal metal concentration.

3.2.3 Results and Discussion

In the present study, the results of the acute manganese toxicity tests expressed by the 48 h EC_{50} values for *C. silvestrii* and *D. magna* were 5.93 mg L^{-1} and 51.66 mg L^{-1}, respectively. Significant differences were observed ($P<0.05$, Tukey's test) in the EC_{50} values for the cladocerans, since *C. silvestrii* was nearly ten times more sensitive to this metal than *D. magna*. The ameliorative effect of *D. magna* to Mn toxicity can probably be related to the different hardness values of the dilution water used, that is: hard water (175 to 225 mg L^{-1} $CaCO_3$) for *D. magna* and soft water (40 to 48 mg L^{-1} $CaCO_3$) for *C. silvestrii*. *D. magna* is an exotic species from freshwater regions with very hard water, whereas *C. silvestrii* is a native species from tropical environments, where the water is commonly classified as soft. In this context, the importance of using species from tropical environments, such as *C. silvestrii,* as reference organisms in toxicity tests was verified, since this species may better represent the toxicity of samples in tropical regions.

The variations in Mn concentration that immobilized 50% of the dafinids in the present study and according to data reported in the literature are presented in Table 1. The variations in Mn toxicity observed for the two species can be related to some chemical characteristics of the water used to dilute the Mn chloride (i.e. different hardness and pH values), and also to characteristics of the different species employed. In general it could be observed that the higher CE_{50} or LC_{50} values reported in the literature for the different species of *Ceriodaphnia*, were related to the higher hardness values. In the present study, the water used in the toxicity bioassays with the tropical cladoceran *C. silvestrii* (soft water=42 to 48 mg L^{-1} $CaCO_3$) could have contributed to a greater availability of the Mn in the tests, since *C. silvestrii* was more sensitive according to the EC_{50} (5.93 mg L^{-1} Mn) than all the other species tested in the different studies (Table 3.2.1).

Lasier et al. (2000) concluded that the tolerance of *Ceriodaphnia dubia* to Mn was related to water hardness, since significant difference was not observed between moderately

hard (92 mg L^{-1}) and hard waters (184 mg L^{-1}), although it was significantly lower in soft waters (26 mg L^{-1}), since the acute LC_{50} values for *C. dubia* averaged 14.5, 15.2 and 6.2 mg L^{-1} Mn, respectively. Further to the influence of hardness on the toxicity of Mn, Reimer (1999), proposed guidelines to protect freshwater life in British Columbia in a review of the organisms. According to the guidelines, for *D. magna* the EC_{50} values progressively increased from 0.8 to 28.7 and to 76.3 mg L^{-1} Mn, when the hardness values registered were 25, 100 and 250 mg L^{-1} $CaCO_3$, respectively, indicating a relationship between the hardness and manganese toxicity. Moreover, Haimer el al. (2015), also concluded that Mn toxicity was higher in slightly soft waters, since the protection guideline for six tropical freshwaters species presented a low value of 73 µg L^{-1}. The cladoceran tropical specie *Moinadaphnia macleayi* was more sensitive to Mn in low hardness water than most of the species cited in the international literature, presenting a 96 h IC_{50} of 1100 µg L^{-1} Mn.

Yim et al. (2006), also confirmed the assumption that an increase in water hardness was related to a decrease in toxicity of the metal, due to competition between the metal and Ca^{2+} and Mg^{2+} ions for binding sites on the cell surfaces of the organisms.

Table 1 shows that the 48h-EC_{50} values for *D. magna* presented variations between the different studies. In the present study an EC_{50} of 51.66 mg L^{-1} was recorded for *D. magna* for a water hardness value of 225 mg L^{-1} $CaCO_3$, in agreement with the results of Raimer (1999) for this specie with compatible water hardness values.

Table 3.2.1 Comparative of EC_{50}/LC_{50} values of manganese for the species *Ceriodaphnia silvestrii*, *Ceriodaphnia dubia* and *Daphnia magna*.

Studies	Test Organism	Prepare of Mn test solutions	pH (mean)	EC_{50}/LC_{50}	Effective Concentration (mg L^{-1})	Hardness (mg L^{-1} $CaCO_3$)
Present study	*Ceriodaphnia silvestrii*	$MnCl_2 . 4H_2O$	7.5	EC_{50}	5.93	44
			8.0		6.2	26
Lasier et al. (2000)	*Ceriodaphnia dubia*	$MnCl_2 . 4H_2O$	8.1	LC_{50}	14.5	92
			8.2		15.2	184
Boucher and Watzin (1999)	*Ceriodaphnia dubia*	Sediment pore water	7.6	LC_{50}	9.1	75-100
Present study	*Daphnia magna*	$MnCl_2 . 4H_2O$	8.0	EC_{50}	51.66	225
					0.8	~25
Reimer (1999)	*Daphnia magna*	-	-	LC_{50}	28.7	~100
					76.3	250
Baird et al. (1991)	*Daphnia magna*	$MnCl_2$	-	mean EC_{50}	28.9	ASTM/hard water
Khangarot and Ray (1989)	*Daphnia magna*	$MnSO_4 . 7H_2O$	7.6	EC_{50}	8.28	240

-Dates not given

As can be seen in Figure 3.2.1, the Mn concentrations recorded in a previous study of samples from the Antas reservoir showed a potential for Mn toxicity rather than the acute toxicity of the present results (EC_{50}) using *D. magna* and *C. silvestrii*. According to the technical report of the water commission (Relatório Técnico da Comissão das Águas, 2012) which evaluated the chemical quality of both the water and sediment of the Ribeirão das Antas river, the samples coming from the Antas reservoir, which receives treated effluents from the UTM/INB, presented high Mn concentrations in both the water (~ 3.6 mg L^{-1} Mn) and sediment (~ 16 g Kg^{-1} Mn), denoting a negative influence of the UTM/INB acid water treatment system on the chemical quality of the Ribeirão das Antas river. The high Mn concentration registered in the sediment (~ 16.000 mg Kg^{-1} Mn) from the Antas reservoir should be considered, since it can affect the aquatic community due to the bioavailability of this metal in the water column on dissolution, and/or due to ingestion by the organisms of particles associated the metal, evidencing the toxic potential of Mn to both zooplankton species at this site.

In a study carried out on daily variations in the Antas reservoir, Ronqui et al. (2010) recorded the highest peak values for manganese at noon (18.2 mg L^{-1} Mn) and at 6pm (20.3 mg L^{-1} Mn) in February in the water samples coming from the UTM/INB site where the treated effluent was discharged, indicating seasonality in the toxicity potential of the water samples towards *C. silvestrii* (EC_{50} = 5.93 mg L^{-1} Mn).

Since the Mn concentrations in the Antas reservoir vary seasonally, as shown by studies carried out in this reservoir, it has been suggested that monitoring of this site be carried out continuously, due to the Mn toxicity potential presented, as observed in the studies by Ronqui et al. (2010) and reported in the technical report of the water commission (Relatório Técnico da Comissão das Águas 2012). In addition, according to previous studies, Mn concentrations above the limit established by current legislation that is 0.1 mg L^{-1} (Conama 2005) have been recorded in the water samples.

Moreover it is important to point out that the sediments can act as sinks for many anthropogenic contaminants, but that, for instance, a lowering of the pH value may remobilize them into the pore-water and then into the water column (Boucher and Watzin 1999; Antunes et al. 2007). Sediments can also register the historical contamination at the location, as observed in the sediment samples taken from the Antas reservoir (Relatório Técnico da Comissão das Águas 2012), thus confirming the need for greater operational control of the treatment system applied to effluents by nuclear installations (UTM/INB).

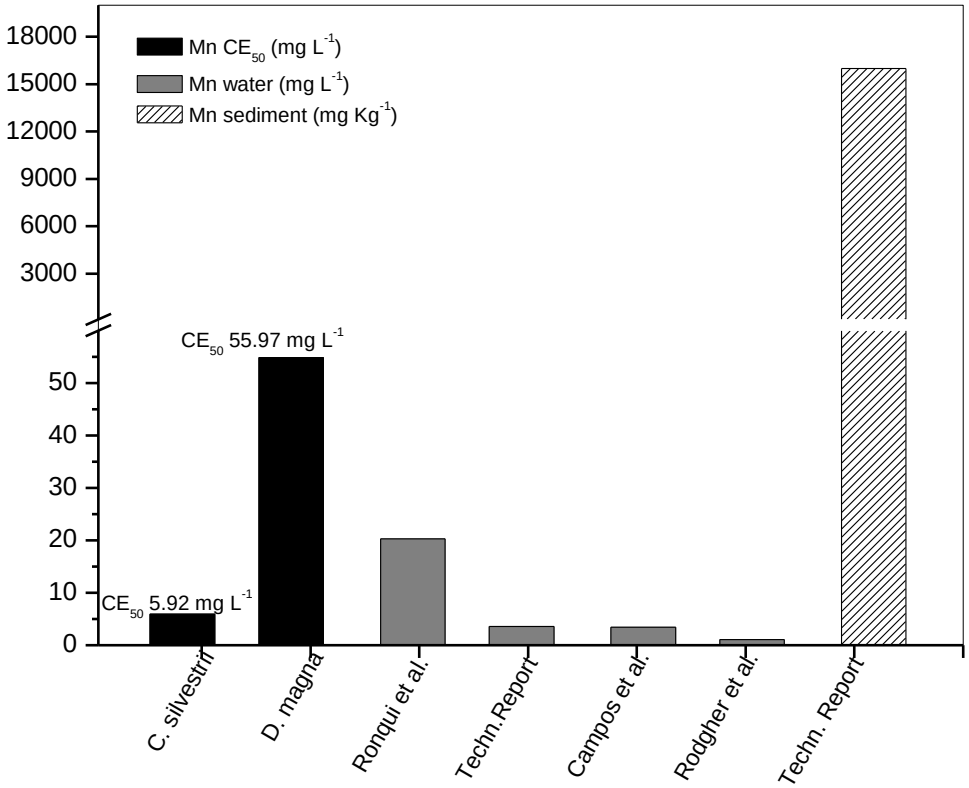

Figure 3.2.1 Mn Concentrations found in previous studies (Ronqui et al. 2010; Techn.Report-Relatório Técnico da Comissção das Águas 2012; Campos et al. 2011; Rodger et al. 2013), carried out on the sediment (mg Kg^{-1}) and water (mg L^{-1}) samples taken at the Antas reservoir and the EC_{50} values (mg L^{-1}) for *D. magna* and *C. silvestrii*.

3.2.4 Conclusions

Since the manganese occurs in the composition of the uranium effluents which may contain other stable and radioactive elements, and the values showed seasonal variations, ecotoxicological monitoring of the water of the Antas reservoir has been suggested, aiming to assess the potential synergistic and antagonistic effects of the chemical mixture that makes up the radioactive effluents that are treated and released into the environment. *C. silvestrii* was more sensitive than *D. magna* to the effects of Mn at the concentrations usually found in the aquatic systems impacted by mineral activities. In order to suggest *C. silvestrii* as a test organism for general applications in toxicity assessments, additional experiments with metal mixtures should be carried out.

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CHAPTER 3.3. An approach to the speciation and acute toxicity of uranium on the Daphnids *Daphnia magna* and *Ceriodaphnia silvestrii*

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Abstract. Acute uranium toxicity tests (48 h) were carried out for *Daphnia magna* and *Ceriodaphnia silvestrii* by exposing each species in specific media at different pH values. The EC_{50} values varied greatly between the two species and the media, that is: for *D. magna* the EC_{50} values ranged from 0.56 to 2.34 mg L^{-1} U at pH 7 and 8, respectively; and for *C. silvestrii* the EC_{50} value was 0.07 mg L^{-1} U at pH 7.5. The present study showed that the pH influenced uranium speciation due to higher or lower concentrations of the toxic uranyl species (UO_2^{2+} and UO_2OH^+), which control the toxicity and hence the bioavailability of the metal to the biota. In addition it was observed that *C. silvestrii* was much more sensitive to uranium toxicity than *D. magnum*, a fact that indicated the importance of using indigenous species, since they represent the local flora.

3.3.1 Introduction

Uranium is an element that occurs naturally in the environment as a mixture of three radioisotopes, that is ^{238}U (99.284%), ^{235}U (0.72%) and ^{234}U (~0.0054%) (Mathews et al. 2009; Mkandawire 2013). Of the three, the ^{234}U and ^{235}U isotopes pose greater radiological toxicity than ^{238}U, due to their shorter half-lives (Mkandawire 2013). On the other hand, the much longer half-life (4.5×10^9 y) of ^{238}U associated with its low specific activity (1.24×10^4 Bq U/g) suggest that large amounts of ^{238}U would have to be ingested for the radiological risk to exceed the chemical risk. According to Sheppard et al. (2005) the main concern in relation to U is that it may exert both chemical and radiological toxicity on terrestrial and aquatic organisms, but its chemical toxicity as a heavy metal is of greater concern. Moreover, due to its considerable tendency to dissolution and transport, U is considered to be one of the most important and potential ecotoxicological concerns to freshwater biota exposed to uranium mine effluent and the mining process (Poston et al.1984; Riethmuller et al. 2001; Antunes et al. 2007).

Uranium may occur in surface waters in different oxidation states: U^{+4} (UIV), UO_2^{+} (UV) and UO_2^{2+} (UVI), and in oxic freshwaters with pH values from 5 to 9, U is present as U (VI) (Markich et al. 2002). In aquatic systems the bioavailability of this radionuclide to the organisms depends on its speciation, which is mainly controlled by variables such as pH, E_H, alkalinity, hardness and dissolved organic matter (Markich et al. 1994; 1996; 2002; Franklin et al 2000; Riethmuller et al. 2001; Sheppard et al. 2005). Once dissolved in freshwater environments uranium may be found in a variety of forms including: the free uranyl ion (UO_2^{2+}), hydrated uranyl ion (UO_2OH^{+}), and also as uranyl complexes bound to inorganic (sulfates, phosphates, carbonates) and organic (humic and fulvic acids) compounds (Gascoyne 1992). According to Markich (2002), the speciation of uranium is relatively complex, but there is reasonable evidence from the literature indicating that UO_2^{2+} and $UO_2OH^{+}$are the major bioavailable forms of U in surface waters. With respect to the aquatic biota the toxicity of uranium is mainly due to the free uranyl ion UO_2^{2+}, with a smaller contribution from uranyl hydroxide UO_2OH^{+} (Markich et al. 2000).

In relation to the acute and chronic ecotoxicological data concerning uranium, a number of studies have shown variations in the sensibility of the freshwater organisms tested, in particular, due to differences in the chemical composition of the medium (e.g. alkalinity, hardness, pH), which are related to the complexation of uranium and to its bioavailability to the organisms. Moreover, according to the literature, the effects of uranium are usually

reported in concentration values, according to the assumption that the chemotoxicity of this radionuclide predominates over its radiotoxicity (Sheppard et al. 2005; Zeman et al. 2008). According to the literature, the majority of the data concerning the toxicity of uranium (CE_{50}) with respect to the cladoceran species used in tests, are mostly for the specie *Daphnia magna* (Poston et al. 1983; Barata et al. 1998; Zeman et al. 2008), followed by *Ceriodaphnia dubia* (Kuhne et al. 2002) and *Moinadaphnia macleayi* (Semaan et al. 2001). On the other hand, there is no available data in the literature concerning the toxicity of U to the neotropical cladoceran *Ceriodaphnia silvestrii*. However it has been suggested that the use of indigenous species may reflect a more realistic test of the locality, since these species are representatives of the local flora (Harmon et al. 2003). Thus the use of indigenous species such as *Ceriodaphnia silvestrii*, the specie used in the present study, has been recommended by water quality regulators.

In this context, the present study aimed to determine the acute toxicity of uranium for *Ceriodaphnia silvestrii* and *Daphnia magna* in a range of concentrations, using synthetic media in laboratory bioassays. In addition, the present study determined the uranium speciation at pH 7 and pH 8 for the exotic specie *D. magna* and at pH 7.5 for the indigenous specie *C. silvestrii*, so as to determine the bioavailability and toxicity of the dominant uranium species for these daphnids.

3.3.2 Materials and Methods

3.3.2.1 Maintenance of cultures and sensibility tests

The *Ceriodaphnia silvestrii* cultures were maintained in continuous parthenogenic reproduction, temperature of 24 ± 2 °C, 16:8 h light/dark photoperiod in an artificial culture medium at pH 7.5 (ABNT 2010). The composition of the ABNT medium was: 0.17 mM Ca, 27 nM Cl, 0.25 mM Mg, 0.42 mM SO_4, 27 µM K, 0.57 mM Na and 0.57 mM HCO_3. The *D. magna* cultures were also maintained in continuous parthenogenic reproduction, temperature of 20 ± 2 °C) 16:8 h light/dark photoperiod in the artificial culture medium M4 (Elendt 1990 modified by ABNT 2009). The compositions of the M4 media at pH 7 and pH 8 were: 1.58 mM Ca, 3.26 mM Cl, 0.4 mM Mg, 0.4 mM SO_4, 0.064 mM K, 0.7 mM Na, 3.6 µM Mn, 14.3 µM Li, 1.16 µM Rb, 1.13 µM Sr, 0.19 µM Cu, 0.19 µM Zn, 83 nM Co, 3 µM NO_3, 46 µM B, 0.15 µM Br, 0.26 µM Mo, 6 nM NH_4, 5 nM V, 19 nM I, 12 nM SeO_3, 35 µM Si, 3.55 µM Fe and 2.1 µM HPO_4.

The daphnids were fed daily on the chlorophycean algae *Raphidocelis subcapitata* maintained in CHU-12 culture medium (Müller 1972). The algae were centrifuged for 15min at 1000g (Sorvall Super T21 centrifuge®) under refrigeration and re-suspended in the appropriate media for *C. silvestrii* and *D. magna*. In the present study the algal concentration provided at each culture renewal ranged from 1 to 5 x 10^5 and 1 x 10^6 cells mL^{-1} per organism for *C. silvestrii* and *D. magna*, respectively, to achieve a daily ration of about 100 ug carbon per organism. In addition, the fish food supplement (Tetramin®), diluted in processed water (Millipore®, model MilliQ gradient) to a concentration of 5 x 10^3 g mL^{-1}, was employed in 40 mL aliquots. Each aliquot was added weekly to a suspension of dry (Fleishmann®) yeast at a concentration of 0.2g per 40mL of processed water, and stored at 4 °C.

According to international and national norms, the 48 h acute toxicity tests with the reference substances $K_2Cr_2O_7$ for *Daphnia magna* (OECD 2004; ISO:6341 2012) and NaCl for *Ceriodaphnia silvestrii* (ABNT 2010) were applied in order to assure that the test conditions were reliable. According to the results of the sensibility tests carried out, the substance NaCl presented an effective inhibitory concentration (48 h EC_{50}) for *C. silvestrii* at average values of 1.13 g L^{-1}. With respect to *Daphnia magna*, average values of 1.44 mg L^{-1} of the substance $K_2Cr_2O_7$ provided an effective inhibitory concentration (48 h EC_{50}). These values for *C. silvestrii* and *D. magna* were within the prescribed ranges set by the guidelines.

3.3.2.2 Uranium solution and exposure conditions

The uranium stock solution was obtained as uranyl nitrate hexahydrate ($UO_2(NO_3)_2.6H_2O$) (Fluka®) which was diluted with deionized water (Milli-Q®) to a concentration of 1 g L^{-1} U in 0.2% nitric acid solution (HNO_3). The uranium test solutions were freshly prepared as from the stock solution using the appropriate medium (M4 for *D. magna* and ABNT for *C. silvestrii*). For all tests, including the controls, in the experiments with *D. magna*, the nitrate concentration was adjusted to 0.15 nM at pH 7 or to 11.95 µM at pH 8, and in the experiments with *C. silvestrii* to 8.91 nM at pH 7.5. This adjustment was done because when the uranium is added as uranyl nitrate hexahydrate one must eliminate the differences in NO_3^- concentrations associated with uranium spikes. It is important to note that the addition of NO_3^- did not affect the survival of the daphnids in the control in any of the tests. The uranium concentrations were determined at the end of all the acute toxicity tests by ICP-MS (Perkin Elmer, model NexIOn 300) applied according to methods 3030E and 3125B, being used to confirm the first and last nominal concentrations tested (APHA 2012), which

remained within 10% of the proposed concentrations. The pH values were monitored at the beginning and end of all tests with all the uranium concentrations tested, and remained within 0.1 unit of the nominal pH value.

3.3.2.3 Acute tests

The acute tests used to determine the 48 h EC_{50} of uranium followed the methodologies according to OECD (2004) and ISO:6341 (2012) for *D. magna* and those of ABNT (2010) for *C. silvestrii*. In the 48 h acute tests for *D. magna* in M4 at pH 7, the uranium concentrations ranged from 0.1 to 1.2 mg L^{-1} and at pH 8 from 1 to 10 mg L^{-1}. On the other hand, in the 48 h acute tests for *C. silvestrii* the U concentrations tested ranged from 0.025 to 0.75 mg L^{-1}.

The pH of the water was adjusted to pH 7.0 ± 0.2 and pH 8.0 ± 0.2 using dilute acid (HCl) and/or base (NaOH). For each condition tested the juveniles (<24-h old) were divided into four groups (replicates) with five animals in each, and exposed in polycarbonate tubes (Brand®) containing 10 mL (*C. silvestrii*) and 50 mL (*D. magna*) medium at each concentration, without renewal. For all the bioassays, the control treatments were prepared using only dilution water. The experimental conditions (temperature and photoperiod) and dilution waters used in these experiments were the same as those used to culture the organisms. After 48 h exposure, the number of mobile animals was counted, considering that, after 15-s, animals showing no response to gentle agitation were considered dead according to the methodologies cited above.

3.3.2.4 U speciation

The U speciation in each of the different test solutions (M4 media at pH 7 and pH 8; ABNT medium at pH 7.5) was calculated using the PHREEQC Interactive 3.3.10-12220 speciation code with the lln.dat thermodynamic database, which includes all relevant uranium chemical species in surface waters as described by Goulet et al. (2015). The input parameters in the PHREEQC to estimate the concentrations of free uranyl ion, and also its complexing ions in the uranium concentrations EC_{50} tests with *D. magna* and *C. silvestrii*, were based on the chemical compositions of the different media used, that is, for *D. magna* the M4 medium at both pH 7 and pH 8, and for *C. silvestrii* the ABNT medium at pH 7.5. The chemical compositions of the M4 media at pH 8 and pH 7 were the same, since they only differed

according to the Cl concentration (added as HCl to reduce the pH value). The values for alkalinity in the different media were measured according to Table 1.

3.3.2.5 Chemical analyses

The total uranium concentrations in the test solutions were analyzed by induced coupled plasma mass spectrophotometry (ICP/MS, Perkin Elmer, model NexIOn 300) with a detection limit of 2.5 µg L^{-1}, following methods 3030E and 3125B according to APHA (2012). The hardness and alkalinity of the cultivation waters were measured in the aqueous samples by titration with EDTA according to methods 2340C and 2320B (APHA 1995).

3.3.2.6 Statistical analysis

The differences in the survival of the cladocerans between the control treatment and the test conditions using different uranium concentrations were determined using the Trimmed Spearman-Karber computer program which estimated the uranium concentration that caused an acute effect in 50% (CE_{50}) of the organisms tested (Hamilton et al. 1977).

3.3.3 Results and Discussion

In aquatic system, be it in natural freshwaters or in laboratory experiments, the uranium speciation is mainly controlled by key physicochemical variables (i.e. alkalinity, hardness, pH and others) which are related to its bioavailability (i.e. the ability of this radionuclide to bind to or traverse the cell surface of an organism, by uptake) and the toxicity for aquatic organisms (Markich and Jeffree 1994; Markich et al. 1996; Markich 2002).

Overall, the speciation calculation indicated that the most dominant U species at pH 8 was $(UO_2)_2(OH)_3CO_3^-$ (6.62 x 10^{-1} mg L^{-1}), and to a lesser extent UO_2OH^+ (3.12 x 10^{-1} µg L^{-1}) and UO_2^{2+} (8.78 x 10^{-4} µg L^{-1}). On the other hand, at pH 7 the speciation of uranium in the toxicity tests showed that the hydroxyl forms were the most important ones, as represented by $UO_2(OH)_2$ (0.151 mg L^{-1}) and UO_2OH^+ (1.56 µg L^{-1}), followed by the free uranyl ion UO_2^{2+} (4.41 x 10^{-2} µg L^{-1}), as shown in Table 4.3.1. For *C. silvestrii* the dominant U species at pH 7.5 was $UO_2(CO_3)_2^{2-}$ (0.37 µg L^{-1}), followed by UO_2OH^+ (9.05 x 10^{-2} µg L^{-1}) and UO_2^{2+} (5.39 x 10^{-4} µg L^{-1}).

The toxicity of uranium for aquatic biota is assumed to be mainly caused by the free uranyl ion UO_2^{2+}, with a lesser contribution by uranyl hydroxide UO_2OH^{+} (Markich et al. 1996; 2000; 2002). In the present study the 48 h acute toxicity tests with *D. magna* in M4-pH 7 and M4-pH 8 showed that the UO_2^{2+} and UO_2OH^{+} species presented variations in their concentrations in both experiments. Once dissolved at pH 7, the free $UO_2^{2+}$and hydrated UO_2OH^{+} presented higher concentrations than at pH 8. In general it was observed that an increase in pH from 7 to 8 was accompanied by decreases in the concentrations of UO_2OH^{+} and UO_2^{2+} in all the tests, by factors of 10 and 100 times, respectively. In all experiments, the pH was monitored at the beginning and end of the tests and remained within 0.2 of a unit of the nominal pH value. Since the hardness remained the same (210 mg L^{-1} $CaCO_3$) and the alkalinity only varied from 27 to 37 mg L^{-1} $CaCO_3$, this suggested that the pH was the most important variable that may have influenced the calculation of the dominant U species and thereby its bioavailability and toxicity EC_{50} values, which were: 0.58 mg L^{-1} U (pH 7) and 2.36 mg L^{-1} U (pH 8) in the experiments using *D. magna*. For *C. silvestrii* in ABNT medium, pH 7.5, water hardness at 47 mg L^{-1} $CaCO_3$ and alkalinity at 32 mg L^{-1} $CaCO_3$, the UO_2^{2+} and UO_2OH^{+} species presented variations of their concentrations in relation to the results obtained for *D. magna* at pH values of 7 and 8. This was expected due to the differences between the test conditions of the media. As from the experimental conditions used in the tests, differences could be observed in the uranium toxicity, that is, for *C. silvestrii* the EC_{50} value 0.07 mg L^{-1} U found was much lower than the EC_{50} values of 0.58 and 2.36 mg L^{-1} registered for *D.magna* at pH 7 and 8, respectively.

According to Riethmuller et al. (2001) and Markich (2013) it is important to separate the effects of the different variables (such as true hardness, alkalinity and pH), since they exhibit different mechanisms that may act on the expression of toxicity. It is therefore suggested that the toxicity mechanisms of the physicochemical variables have not yet been completely elucidated, since differences in the understanding of these mechanisms with respect to the bioavailability and toxicity of uranium have been observed. Moreover Makndawire (2013) emphasized the fact that the assessment of U toxicity is rare and controversial, due to the fact that most organisms display intrinsic hermetic responses to low concentrations and chronic exposure. Thus with a view to determining how the toxicity to organisms in surface freshwaters is influenced by water quality, some studies have investigated the effects of one or more variables as possible modifiers of uranium toxicity (Poston et al. 1984; Barata et al.1998; Franklin et al. 2000; Riethmuller et al. 2001; Charles et al. 2002; Zemann et al.2008; Markich et al. 2013; Goulet et al. 2015).

Franklin et al. (2000) used bioassays to determine the toxicity of U using freshwater alga (*Chlorella* sp.) in a synthetic soft-water medium. They verified that the toxicity of this radionuclide was highly pH-dependent, that is, at pH 5.7 and pH 6.5 the 72 h EC_{50} values were, respectively, 78 and 44 µg L^{-1} U. In addition the authors verified that differences in the concentrations of the free metal ion (UO_2^{2+}) in the calculation of U speciation were minimal (< 10%) at the pH values of 5.7 and 6.5, suggesting that a mechanism of competition between H^+ ions and the metal ion at the cell surface was more relevant than metal speciation in this case. Differently to that observed by Franklin et al. (2000), in the tests carried out with *D.magna* in the present study, the differences in speciation calculated in relation to the free uranyl ion specie (UO_2^{2+}) were highly relevant, ranging from 8.78 x 10^{-4} to 4.41 x 10^{-2} µg L^{-1} at pH 8 and pH 7, respectively, indicating that the pH value was very important in U speciation. In addition, under the same pH conditions presented by Franklin et al., that is, 5.7 and 6.5, differences were also verified in the speciation of UO_2^{2+}, indicating that the water quality influenced the uranium speciation in the present study.

In a study carried out by Zeman et al. (2008) with *D. magna* at pH 7 and pH 8 using the M4 synthetic medium, they concluded that reducing the pH (from 8 to 7) and alkalinity, increased the proportion of UO_2^{2+}, resulting in a decrease in the 48 h LC_{50} values from 7.8 to 0.39 mg L^{-1} U, respectively. In the present study, as also observed by Zeman et al. (2008), an increase in the UO_2^{2+} concentration from 8.78 x 10^{-4} at pH 8 to 4.41 x 10^{-2} at pH 7 was verified, resulting in a decrease in the 48 h EC_{50} from 2.4 to 0.56 mg L^{-1} U, respectively. It is worth mentioning that, differently from the experiment of Zeman et al. (2008), in the present study the alkalinity varied little, from 27 to 37 mg L^{-1} $CaCO_3$ at pH 7 and pH 8, respectively, in the toxicity tests carried out with *D. magna*.

Table 3.3.1 gives a summary of values obtained for EC_{50} in uranium toxicity tests carried out at different pH values with daphnid species. The results obtained for acute uranium toxicity for *D. magna* at pH 8 were much lower than at pH 7, giving average 48 h EC_{50} values of 2.36 and 0.58 mg L^{-1} U, respectively. The results obtained for EC_{50} indicated that *Daphnia magna* was more sensitive at pH 7 than at pH 8, probably due to the dominant uranium species. On the other hand, for *Ceriodaphnia silvestrii* the EC_{50} value of 0.07 mg L^{-1} recorded was approximately ten orders of magnitude lower than the EC_{50} value of 0.58 mg L^{-1} recorded for *D. magna* at pH 7, indicating that this indigenous species was much more sensitive than *D. magna* to acute uranium toxicity. The differences observed between *C. silvestrii* and *D. magna* in the 48 h EC_{50} acute toxicity tests may have been related to the water hardness specific for each species, that is, 47 and 215 mg L^{-1} $CaCO_3$, respectively.

Table 3.3.1 Relationship of the water quality with the uranium speciation and acute toxicity of this metal for the species *D. magna* and *C. silvestrii* in the present study and according to the literature.

		This study[a,b]			**Zeman et al. (2008)**[a]		**Barata et al. (1998)**[a]	
		D. magna		***C. silvestrii***	***D. magna***		***D. magna***	
Water Quality	EC_{50} U (mg L^{-1} U)	0.49<0.56<0.69	2.06<2.4<2.79	0.05<0.07<0.08	0.39	7.8	8.3	22.4
	Alkalinity (mg L^{-1} $CaCO_3$)	27	37.7	32	2.7	34	62	126
	Hardness (mg L^{-1} $CaCO_3$)	215	215	47	250	250	90	179
	pH	7	8	7.5	7	8	7.7	8.1
Speciation U (VI)	UO_2^{2+} (µg L^{-1})	4.41×10^{-2}	8.78×10^{-4}	5.39×10^{-4}	1.7×10^{-1}	1.7×10^{-4}	7.0×10^{-3}	1.5×10^{-3}
	UO_2OH^+ (µg L^{-1})	1.56	3.12×10^{-1}	9.05×10^{-2}	-	-	-	-
	$(UO_2)_2(OH)_3CO_3^-$ (mg L^{-1})*		6.62×10^{-1}		-	-	-	-
	$UO_2(OH)_2^0$ (mg L^{-1})*	1.51×10^{-1}			-	-	-	-
	$UO_2(CO_3)_2^{2-}$ (µg L^{-1})*			0.37	-	-	-	-

[a] Studies that used syntetic water.

[b] CE_{50} average values and confidence intervals.

*Main forms U in speciation calculation in this study.

In a study carried out with a freshwater macrophyte (*Ceratophyllum demersum*) in a synthetic medium, Markich (2013) verified that the U speciation calculations revealed negligible changes in the proportion of key U species (UO_2^{2+} UO_2OH^+ or $UO_2(OH)_2$ in the range of water hardness values tested (20, 75, 150, 275 and 400 mg L^{-1} $CaCO_3$). According to the author a 20-fold increase in water hardness (from 20 to 400 mg L^{-1} $CaCO_3$) resulted in a 4-fold decrease in U toxicity, that is, the median values for EC_{50} ranged from 547 µg L^{-1} to 134 µg L^{-1}. Moreover Charles et al. (2002) also confirmed the ameliorative effect of water hardness on the U toxicity against the green alga *Chorella* sp., where a 50-fold increase in water hardness (from 8 to 400 mg L^{-1} $CaCO_3$) resulted in a 5-fold decrease in U toxicity, that is, the 72 h EC_{50} increased from 56 to 270 µg L^{-1} U, respectively, for this freshwater alga. In addition they also verified that the calculation of U speciation showed there were no significant (*P>0.05*) differences in the predicted U speciation amongst the four water hardness levels tested (8, 40, 100 and 400 mg L^{-1} $CaCO_3$). Riethmuller et al. (2001) postulated that uranium toxicity was inversely related to an increase in water hardness, where the cations Ca^{2+} and Mg^{2+} may have competitively inhibited the uptake of UO_2^{2+} on the cell surface of *Hydra viridissima*. Furthermore, to the contrary, these same authors verified that a 25-fold increase in alkalinity (from 4 to 102 mg L^{-1} $CaCO_3$) in permanent hardness (165 mg L^{-1} $CaCO_3$), did not affect the EC_{50} U toxicity (177 and 171 mg L^{-1} U, respectively) for *H. viridissima*. Yim et al. (2006) also confirmed the assumption that an increase in water hardness was related to a decrease in toxicity of a metal mixture, due to competition between the metals and the Ca^{2+} and Mg^{2+} ions for binding sites on the cell surfaces of the organisms.

Semaan et al. (2001) carried out acute and chronic U toxicity tests using different populations (some maintained in the laboratory and other wild populations collected in the environment) of the tropical cladoceran *Moinadaphnia macleayi* and verified there were little differences in the responses between these different populations concerning acute and chronic exposure. The 48 h EC_{50} values ranged between 0.16 - 0.39 mg L^{-1} U for the *M. macleayi* populations, which were thus shown to be significantly more sensitive than the other species previously tested. In the present study the *Ceriodaphnia silvestrii* tropical specie was even more sensitive than *Moina macleayi*, with a 48 h EC_{50} value of 0.07 mg L^{-1} U.

Pickett et al. (1993) carried out a study to determine the acute and chronic toxicities of U compounds (uranyl nitrate, uranyl hydrogen phosphate and the free uranyl ion) in low hardness waters (3 mg L^{-1} $CaCO_3$) for the organism *Ceriodaphnia dubia*. The LC_{50} for the three compounds ranged from 0.05 - 0.10 mg L^{-1} U in the chronic U toxicity tests (7-d), and *C. dubia* proved to be highly sensitive according to the NOEC and LOEC values, which

ranged from <0.006-0.021 mg L^{-1} U and from 0.006-0.038 mg L^{-1} U, respectively. Comparing the results of the present study with those of Pickett et al. (1993), *Ceriodaphnia silvestrii* was as sensitive as *Ceriodaphnia dubia* in the uranium toxicity tests. On the other hand, the results of the present study showed that *Daphnia magna* was less sensitive than the *Ceriodaphnia* species used in different studies reported in the literature.

Kuhne et al. (2002), analyzed the effects of depleted uranium toxicity on the survival (96-h acute test) and reproduction (7-d chronic test) of *Ceriodaphnia dubia*, as from the use of test water (prepared as from test soil and reference soil containing water obtained from a well) with the following characteristics: hardness of 182 mg L^{-1} $CaCO_3$, alkalinity of 145 mg L^{-1} $CaCO_3$ and a pH value of 7.87. According to the authors, the median lethal concentration (LC_{50}) was 10.5 mg L^{-1}, whereas the reproduction effects were observed at ≥3.91 mg L^{-1} and 1.97 mg $L^{-1}$for LOEC and NOEC, respectively. In the present study the toxicity results observed for *C. dubia* were very different from those obtained by Pickett et al. (1993) and Kuhne et al. (2002). Such differences in relation to the sensitivity of this specie may be related to the hardness of the water used and/or the alkalinity. Thus in the present studies the U toxicity for *C. dubia* was shown to decrease about 100-fold with a 60-fold increase in water hardness. According to Markich (2013) an increase in hardness may inhibit the U toxicity through the binding/uptake of Ca^{+2} and/or Mg^{+2} cations at the cell surfaces of the organisms.

Assuming that the toxicity of U is dependent on its speciation and on physicochemical variables, and that in the toxicity experiments with *D.magna* the pH only ranged from 7 to 8 (alkalinity varied very little and hardness remained constant), some assumptions were made: i) at pH 7 the U speciation was dominated by the main cationic species of biological concern, that is, UO_2OH^+ (1.56 µg L^{-1}) and UO_2^{2+} (4.41 x 10^{-2} µg L^{-1}), which, in turn, probably controlled the bioavailability and toxicity of uranium for the *D. magna* organisms; ii) at pH 8 the uranium speciation showed that the uranyl hydroxide carbonate specie $[(UO_2)_2(OH)_3CO_3]^-$ (6.62 x 10^{-1} mg L^{-1}) was the most important, while the species of biological concern, UO_2OH^+ (3.12 x 10^{-1} µg L^{-1}) and UO_2^{2+}(8.78 x 10^{-4} µg L^{-1}), were present in lower concentrations than observed at pH 7, hence indicating reduced bioavailability and toxicity for *D. magna*, according to the EC_{50} uranium values registered.

3.3.4 Conclusions

Overall, according to the observations reported in the literature and the results obtained in the present study, it is suggested that negligible variations in uranium speciation

are observed when the pH is maintained constant and the hardness increased, and in this case, the U toxicity and bioavailability are controlled by competition between other cations present in the medium together with the uranium species, thus influencing the uptake of U by the organisms. In contrast, when the hardness is maintained constant and the pH altered, changes in uranium speciation may be observed, which will control the toxicity of the metal due to higher or lower concentrations of the toxic uranyl species. Furthermore, it is important to point out that uranium toxicity may also be influenced by the type of organism tested, since they can show different responses, for instance, some organisms have the ability to relocate their resources (i.e., energy supply or multiplying rapidly) to withstand low concentrations of U toxicity (Makndawire 2013).

Thus, additional studies are required to accurately access the different key physicochemical variables which may act on the expression of uranium toxicity. In the present study the pH was shown to influence uranium speciation, and hence the bioavailability and toxicity of U for *D. magna*. In addition it was observed that *C. silvestrii* was much more sensitive to uranium toxicity than *D. magna*, a fact that indicated the importance of using indigenous species, since they represent the local flora.

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CHAPTER 3.4 Evaluation of the treatment system of the uranium mine effluent, from the chemical characterization and acute toxicity potential carried out using *Ceriodaphnia silvestrii* and *Daphnia magna*.

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Abstract. The first uranium mine in Brazil was formed as a result of the exploration of uranium deposits in the grounds of the Mineral Ore Treatment Unit of the Brazilian Nuclear Industries (UTM/INB). Currently this open uranium pit mine (PM pond) is being filled with radioactive acid effluent produced continuously by acid mine drainage (AMD), which, after chemical treatment, is deposited in a pond (P41-S), where the stable and radioactive metals are submitted to a precipitating process, and then discharged into the environment, that is, into the Antas reservoir. The present study evaluated the efficiency of the system used to treat the uranium mine effluent by way of a chemical characterization and also an evaluation of its acute toxicity potential using *Ceriodaphnia silvestrii* and *Daphnia magna*. The PM pond presented high concentrations of several chemical species (i.e. Al (54.1 mg L^{-1}), Mn (29.17 mg L^{-1}), Zn (4.46 mg L^{-1}), U (1.65 mg L^{-1}), F^- (12.46 mg L^{-1}) and Th (0.19 mg L^{-1}), being considered acutely toxic, since the average EC_{50} values obtained in this pond for *C. silvestrii* and *D. magna* were: 0.34<0.41%<0.52 and 4.77<5.41%<6.18, respectively. In addition samples taken from the P41-S pond were also considered acutely toxic for both daphnids, since they also presented high concentrations of some chemical species such as F^- (15.2 mg L^{-1}) and SO_4^{2-} (428.5 mg L^{-1}), besides showing high values for electrical conductivity (1137.75 mg L^{-1}) and hardness (799.6 mg L^{-1}) as compared to samples taken from the reference site (CAB point) at the Antas reservoir, which did not present significant differences ($P>0.05$) in their results for toxicity, when compared to the control treatments. The results of the present study, as well as those of the physicochemical species, indicated that the process used to treat the *in natura* effluent of the PM pond, as carried out by the mining company (UTM/INB) was improper and inefficient, since the treated effluent samples taken from the P41-S pond were as toxic for both daphnids as the *in natura* effluent samples taken from the PM pond.

3.4.1 Introduction

Mining activities involve great environmental modifications of the landscape's outline, chemistry and biology, but the main environmental problems resulting from these activities vary depending on the material extracted, processing techniques used, local geography and geological composition (Oliveira and Ávila 2001; Lesley, 2005). In particular, abandoned mines, inactive ones and those in the process of decommissioning require continuous control and monitoring, being considered of great concern (Oliveira and Ávila 2001). It is well known that mining activities may also negatively affect the local biota of freshwater bodies near these areas, modifying their natural community structure and reducing the biodiversity (Ciszewski et al. 2013; Durán et al. 2013; Holopainen et al. 2003; Lefcort et al. 2010).

In addition an important water pollution problem at mining sites is the occurrence of acid mine drainage (AMD), considered to be one of the most significant environmental challenges facing the world, due to the volume of effluents continuously produced (Sheoran and Sheoran 2006). AMD occurs when metal sulfides are exposed to atmospheric, hydrological and biological weathering (i.e. oxygen, water and chemoautotrophic bacteria). The low pH contributes to the mobilization and increase in velocity of the dissolution of some metallic elements (e.g. Al, Cu, Mn, Fe, Cd, Pb, Zn, U), depending on the tailings and waste rock mineralogy (Campos et al. 2011; Luís et al. 2009; Lyew and Sheppard 1997, 2001; Sheoran and Sheoran 2006; Soucek et al. 2001). According to Santos and Ladeira (2011) AMD is considered to be one of the main environmental impacts related to mining activities, due to its dynamics and persistence.

The occurrence of AMD is very common at uranium mining sites because the majority of U ore is associated with pyrite (FeS_2), which, under favorable environmental conditions (exposure to water and air) leads to the formation of H_2SO_4 and $FeSO_4$ and then to $Fe(OH)_3$, the latter being responsible for the reddish or orange color that indicates that AMD is taking place (Mkandawire 2013). AMD produces a large amount of radioactive effluent, which is composed mainly of a highly toxic complex metal mixture. Furthermore U is ubiquitous in natural waters with concentrations that can range from 00.2 to 6 μg L^{-1} U (WHO 2001). Thus although U is considered to be an ubiquitous element, anthropogenic activities such as uranium mining and processing may also lead to an enrichment of the metals and U in natural waters and rivers downstream from the locality where the effluents are discharged, due to their improper disposal (Elbaz-Poulichet et al. 1999).

According to Liber et al. (2011), uranium mine effluents commonly contain high concentrations of some heavy metals and metalloids, including radionuclides from waste rock piles and tailing processing, that may cause an increase in the toxic concentrations of these elements in aquatic environments that are receivers of these treated effluents. Hence these acidic effluents, naturally enriched with heavy metals and radionuclides, are the main legacy of uranium mining sites, and one of the main challenges is related to assessing the toxicity of this complex mixture of chemical species (Bessa et al. 2016).

In this context, the first uranium mine in Brazil (Poços de Caldas, Minas Gerais State), was formed as a result of exploring uranium deposits in the grounds of the Mineral Ore Treatment Unit of the Brazilian Nuclear Industries (UTM/INB). Currently AMD is the main environmental liability occurring in this uranium mine, representing an obligation and legal responsibility of UTM/INB with respect to the treatment, decommissioning and recovery of the site. AMD is treated using chemical processes with calcium hydroxide and oxide (slaked lime) in order to increase the pH and precipitate the stable and radioactive metals. After treatment the treated effluent is discharged into the environment at the Antas reservoir.

Thus the aims of the present study were to evaluate the efficiency of the treatment system of the uranium mine effluent from its chemical characterization, and also evaluate its acute toxicity potential using the daphnid species *Ceriodaphnia silvestrii* and *Daphnia magna*, which have been widely used for this purpose. Thus bioassays were carried out with the *in natura* and treated effluent samples from the two ponds of the aquatic system of this uranium mine (UTM/INB). The acute toxicity and water chemical quality of a reference site in the Antas reservoir were also determined.

3.4.2 Material and Methods

3.4.2.1 Study site and sampling

The Ore Treatment Unit of the Brazilian Nuclear Industries (UTM/INB) was the first uranium deposit to be exploited in Brazil. The UTM/INB is located on the Poços de Caldas Plateau (Minas Gerais State, Brazil) at 1291 m above sea level (Figure 1). This area presents a tropical high altitude climate with two distinct seasons: the rainy season, between October and March, which has an average temperature of 20 °C, while the dry season, between April and August, has an average temperature of 14 °C (Rodgher et al. 2013). Uranium production from this mining site started in 1982 and lasted for 13 years, generating a total of 1241 tons of U

(Majdalani and Tavares, 2001). The open uranium pit mine has been filled with radioactive acid effluent, produced continuously by AMD which originated mainly in the depleted uranium waste rock piles. The AMD is currently treated by chemical processes with calcium hydroxide and oxide (slaked lime) and barium chloride, in order to increase the pH values and precipitate the stable and radioactive metals (Nóbrega et al. 2008). After the physicochemical treatment, the effluent is dumped in the P41-S pond, where the stable and radioactive metals finalize the precipitating process, after which it is discharged into the environment at the Antas reservoir.

The UTM/INB effluent samples were collected from the following points as follows: from the pit mine itself, namely PM (corresponding to the uranium mine *in natura* effluent) - these samplings were carried out in February, April and July 2016; and from P41-S (corresponds to treated effluent before being discharged into the environment) - these samples were taken in November 2014, and February, April and July 2015. From the Antas reservoir, water samples were taken at a reference site, that is, at the CAB point (located at the Antas reservoir, upstream of the mine effluent discharge point) also in November 2014 and February, April and July 2015. The water samples for the 48 h acute toxicity tests and physical chemical analyses were collected in 5-L Van Dorn bottles, transferred to previously-washed plastic bottles and stored at 4 °C, until the moment of analysis.

3.4.2.2. Culture maintenance and sensitivity tests

The *Ceriodaphnia silvestrii* cultures were maintained in continuous parthenogenic reproduction at a temperature of 24 ± 2 °C, with a 16:8 h light/dark photoperiod in an artificial culture medium at pH 7.5 (ABNT 2010). The *D. magna* cultures were also maintained in continuous parthenogenic reproduction, but at a temperature of 20 ± 2 °C, with a 16:8 h light/dark photoperiod in the artificial culture medium M4 (Elendt 1990). The daphnids were fed daily on the chlorophycean algae *Raphidocelis subcapitata* maintained in CHU-12 culture medium (Müller 1972). The algae were centrifuged for 15min at 1000g (Sorvall Super T21 centrifuge®) under refrigeration and re-suspended in the appropriate media for *C. silvestrii* and *D. magna*. In the present study the algal concentration provided at each culture renewal ranged from 1 to 5 x 10^5 and 1 x 10^6 cells mL^{-1} per organism for *C. silvestrii* and *D. magna*, respectively, to achieve a daily ration of about 100 ug of carbon per organism. In addition, the fish food supplement (Tetramin®), diluted in processed water (Millipore®, model MilliQ gradient) to a concentration of 5 x 10^3 g mL^{-1}, was provided in 40

mL aliquots. Each aliquot was added weekly to a suspension of dry (Fleishmann®) yeast at a concentration of 0.2g per 40mL of processed water, and stored at 4 °C. According to international and national norms, the 48 h acute toxicity tests with the reference substances $K_2Cr_2O_7$ for *Daphnia magna* (OECD 2004; ISO:6341 2012) and NaCl for *Ceriodaphnia silvestrii* (ABNT 2010) were applied, in order to assure that the test conditions were reliable. According to the results of the sensitivity tests carried out, the substance NaCl presented an effective inhibitory concentration (48 h EC_{50}) for *C. silvestrii* at average values of 1.13 g L^{-1}. With respect to *Daphnia magna*, average values of 1.44 mg L^{-1} of the substance $K_2Cr_2O_7$ provided an effective inhibitory concentration (48 h EC_{50}). These values for *C. silvestrii* and *D. magna* were within the prescribed ranges set by the guidelines.

3.4.2.3 Acute toxicity tests

The 48 h acute toxicity tests using *C. silvestrii* (ABNT 2010) and *D. magna* (ISO 6341:2012; OECD 2004) as the test organisms were carried out with samples from the two UTM/INB ponds, corresponding to the *in natura* effluent (PM) and treated effluent (P41-S). Also, the acute toxicity was determined at a reference site at the Antas reservoir (CAB).

Bioassays were only carried out for the *in natura* effluent samples (PM pond) to set an appropriate dilution range to obtain EC_{50} values with the best confidence intervals. The *in natura* effluent samples were diluted in ABNT and M4 medium for the species *C. silvestrii* and *D. magna*, respectively, to obtain the EC_{50} values as a percentage. For *C. silvestrii* the following *in natura* effluent dilutions were used: 0.10, 0.15, 0.25, 0.40, 0.50 and 0.75%; and for *D. magna* the dilutions established were 2, 3, 5, 6, 7 and 8%. On the other hand, the samples from the (P41-S) pond and the reference site (CAB) were not diluted and used pure (100% sample).

For all the tests, animals younger than 24 hours old were divided into four groups (replicates) of five animals each, and exposed to 10 mL (*C. silvestrii*) and 50 mL (*D. magna*) of each sample in polycarbonate beakers (Brand®). For all the bioassays, control treatments were prepared with the ABNT and M4 medium dilution water. The experimental conditions (temperature and photoperiod) and dilution waters used in these experiments were the same as those used to culture the organisms. The pH values (Micronal®, model B374 potentiometer), electrical conductivity (Orion®, model 145A conductivimeter) and dissolved oxygen (WTW®, model OXI 316i environmental oximeter) of the effluents and water samples tested were determined at the beginning and end of all the acute toxicity tests.

3.4.2.4 Chemical and physical analyses

The electrical conductivity, dissolved oxygen and pH values were measured using a Horiba® model U-10 multiparameter probe field meter. The reduction-oxidation potential was determined using a Digimed® model DM-22 Pt electrode (with Ag/AgCl as the reference). The water hardness (Ca^{2+} and Mg^{2+}) and the metal contents were quantified by inductively-coupled plasma atomic-absorption optical emission spectrometry (Varian®, model Liberty RL). Fluoride was estimated potentiometrically with an ion-selective electrode and sulfate by UV-Vis spectrophotometry (Varian®, model Cary 50) (ASTM 1980). The water samples taken for the metal determinations were preserved by adding nitric acid to pH < 2 (Merck®, Ultrapure acid) and storing at 4 ^{o}C. The total metal concentrations (Ag, Al(d), As, B, Ba, Be, Cd, Co, Cr, Cu(d), Fe, Fe(d), F^{-}, Hg, Li, Mn, Mo, Ni, Pb, Sb and Se), were determined according to the U.S. EPA (2007) method 6010C and measured by atomic emission spectrometry (Varian®, model Libert RL). The uranium concentrations were measured using induced coupled plasma mass spectrophotometry (ICP-MS, Perkin Elmer, model NexIOn 300) following methods 3030E and 3125B according to APHA (2012).

In addition the chemical and physical data obtained for the water sampled from the Antas Reservoir (CAB point) and the treated effluent (P41-S pond) were compared with the limits adopted by the following Brazilian guidelines: Conama Resolution 357 Class II and Conama Resolution 430, respectively.

3.4.2.5 Statistical analyses

The results of the physical and chemical analyses were analyzed using ANOVA and Tukey's test (post hoc test) to detect significant differences between the water samples obtained from the different sites at the Antas reservoir (CAB) and UTM/INB (P41-S and PM ponds) at the different collection times.

The toxicity tests for the water samples collected at CAB and P41-S were analyzed statistically using the BioEstat 4.0 program (Ayres et al 2005) Fisher's exact test was used to distinguish significant differences in the survival of the cladocerans between the control treatment and the treated effluent water samples (P41-S) and in the Antas reservoir (CAB). On the other hand for samples corresponding to the *in natura* effluent (PM), differences in the survival of the cladocerans between the control treatment and the different dilutions of the *in natura* effluent were determined using the Trimmed Spearman-Karber computer program,

which estimated the uranium concentration that caused an acute effect in 50% (CE_{50}) of the organisms tested (Hamilton et al. 1977).

3.4.3 Results

At UTM/INB, the samples from the PM and P41-S ponds presented higher concentrations of chemical species in relation to the samples from the reference site (CAB point at the Antas reservoir) (Table 3.4.1). The PM pond presented high average concentrations of chemical species for all the periods evaluated, especially for: Fe (7.25 mg L^{-1}), Al (54.1 mg L^{-1}), Mn (29.17 mg L^{-1}), Zn (4.46 mg L^{-1}), U (1.65 mg L^{-1}), SO_4^{2-} (356 mg L^{-1}), F^- (12.46 mg L^{-1}) and Th (0.19 mg L^{-1}), and also for electrical conductivity (1765 µS cm^{-1}) and hardness (868.33mg L^{-1}), and an average acid pH value (2.87). The P41-S pond also showed high concentrations of some chemical elements such as: F^- (15.2 mg L^{-1}) and SO_4^{2-} (428.5 mg L^{-1}), as well as high values for electrical conductivity (1137.75 mg L^{-1}) and hardness (799.6 mg L^{-1}), and an average alkaline pH value (8.19).

According to the results obtained with ANOVA for the three sample points, significant spatial differences were revealed for some physicochemical variables. The PM pond was considered statistically different *($P<0.05$)* in relation to the other two points, P41-S and CAB. The average values found for the metals Al (54.1 mg L^{-1}) and Cd (0.013 mg L^{-1}) in the water samples collected from the PM pond, were significantly higher (*$P<0.05$*) than the values determined for the same metals in the water samples from the CAB point, which were 0.20 mg L^{-1} and 0.001 mg L^{-1}, respectively, and the P41-S pond, which were 0.99 mg L^{-1} and 0.001 mg L^{-1}, respectively. Similarly, the samples obtained from the PM pond also presented higher average value for water hardness, that is, 868.33 mg L^{-1} as compared to the value registered at the CAB point of 112.68 mg L^{-1} (*$P<0.05$*). Also for the total iron content, the samples from the PM pond presented a higher average value (7.25 mg L^{-1}) than the values registered for the P41-S pond (0.62 mg L^{-1}) and CAB point (0.82 mg L^{-1}) *($P<0.05$)*. Lower mean values were registered for Ni at the CAB and P41-S points (0.01 mg L^{-1}) and also for Mn, of 0.54 mg L^{-1} 0.80 mg L^{-1}, respectively, as compared to the average values obtained in samples from the PM pond for Ni (0.05 mg L^{-1}) and Mn (29.17 mg L^{-1}) (*$P<0.05$*). The average levels for Th (0.19 mg L^{-1}) and U (1.65 mg L^{-1}) registered in samples from the PM pond were significantly higher than the mean values registered for samples from the CAB point, which were 0.003 mg L^{-1} (for both metals) and from the P41-S pond which were 0.003 mg L^{-1} (Th) and 0.03 mg L^{-1} (U) (*$P<0.05$*). The results referring to the other chemical elements of geochemical and

ecotoxicological concern, such as Ag, B, Ba, Be, Cl-, Co, Cr, Cu(d), Hg, Li, Mo, Pb, Sb and Se did not present significant differences between the three sites evaluated (*P>0.05*).

The present study showed that the *in natura* effluent samples from the PM pond were considered acutely toxic for *D. magna* and *C. silvestrii*, according to the acute toxicity preliminary tests (100%, 50%, 25% and 12.5%), with 0% of mobility being registered for both daphnid species. The *in natura* effluent from the PM pond was able to cause acute toxicity with a very low percentage, since the average values obtained for the 48 h EC_{50} with 95% confidence intervals, for *C. silvestrii* and *D. magna* were: 0.34<0.41%<0.52 and 0 4.77<5.41%<6.18, respectively (Table 3.4.2).

Table 3.4.1 Average values obtained for the physicochemical variables (hydrogenionic potential= pH, redox potential= Eh, electrical conductivity= EC, dissolved oxygen= DO, hardness= water hardness, silver= Ag, Al(d)= dissolved aluminum, arsenic= As, boron= B, barium= Ba, beryllium= Be, cadmium= Cd, chloride= Cl^-, cobalt= Co, chromium= Cr, dissolved copper= Cu(d), iron= Fe, dissolved iron= Fe(d), fluoride= F^-, mercury= Hg, lithium= Li, manganese= Mn, molybdenum= Mo, nickel= Ni, lead= Pb, antimony= Sb, selenium= Se, sulfate= SO_4^{2-}, thorium= Th, uranium= U and zinc= Zn) and the standard

Variables	*in natura* Effluent PM (n=3)	Treated Effluent P41-S (n=4)	Conama/430 Discharged Effluent (2011)	Reference site CAB (n=4)	Conama/357 Class II (2005)
pH	2.87 ± 0.58	8.19 ± 1.00	5-9	6.74 ± 0.93	6-9
Eh	-	198.5 ± 70.09	-	259.75 ± 63.51	-
EC	1765 ± 10	1137.75 ± 243.69	-	199.25 ± 316.40	-
DO	-	4.65 ± 1.32	-	5.36 ± 1.32	≥ 6.0
Hardness	868.33 ± 98.34	799.61 ± 243.57	-	112.68 ± 201.69	-
Ag	0.002 ± 0.003	0.005 ± 0.004	0.10	0.005 ± 0.004	0.01
Al(d)	54.1 ± 26.5	0.99 ± 0.77		**0.20 ± 0.08**	0.10
As	0.0025	0.0025	0.50	0.0025	0.01
B	0.11 ± 0.16	0.04 ± 0.02	5.0	0.04 ± 0.03	0.50
Ba	0.017 ± 0.015	0.02 ± 0.01	5.0	0.02 ±0.01	0.70
Be	0.013 ± 0.006	0.009 ± 0.003	-	0.009 ± 0.003	0.04
Ca	334.13 ± 97.44	319.13 ± 97.26		43.94 ± 82.7	
Cd	0.013 ± 0.004	0.001	0.20	0.001	0.001
Cl^-	3.1 ± 0.2	3.26 ± 1.29	-	5.7 ± 3.3	250
Co	0.023 ± 0.006	0.03 ± 0.01	-	0.03 ± 0.01	0.05
Cr	0.04	0.03 ± 0.02	-	0.028 ± 0.015	0.05
Cu(d)	0.01	0.02 ± 0.02	1.0	**0.023 ±0.024**	0.009
Fe	7.25 ± 3.28	0.62 ± 0.83	-	0.82 ± 0.64	-
Fe(d)	4.29 ± 3.69	0.037 ± 0.006	15.0	**0.41 ± 0.26**	0.30
F^-	12.46 ± 11.17	15.2 ± 11.5	**10.0**	0.96 ± 0.53	1.40
Hg	0.0005	0.0002	0.01	0.0002	0.0002
Li	0.1	0.09 ± 0.03	-	0.088 ± 0.025	2.5
Mg	6.9 ± 0.9	0.58 ± 0.21		0.58 ± 0.27	
Mn	29.17 ± 4.92	0.8 ± 0.6	1.0	**0.54 ± 0.39**	0.10
Mo	0.08 ± 0.11	0.054 ± 0.097	-	0.05 ± 0.09	-
Ni	0.05 ± 0.01	0.01	2.0	0.01	0.025
Pb	0.016 ±0.007	0.01	0.50	0.01	0.01
Sb	0.002 ± 0.003	0.004 ± 0.002		0.003 ± 0.002	0.005
Se	0.005	0.005	0.30	0.005	0.01
SO_4^{2-}	356 ± 606.23	428.5 ± 310.1	-	83.3 ± 159.1	250
Th	0.19 ± 0.04	0.0025	-	0.0025	-
U	1.65 ± 0.11	0.035 ± 0.089	-	0.003 ± 0.002	0.02
Zn	4.46 ± 0.71	0.72 ± 1.38	5.0	0.04 ± 0.04	0.18

Table 3.4.2 Comparison of the EC_{50} values in percentage, as well as the confidence intervals for the daphnids, and of the main chemical elements (mg L^{-1}) registered in the uranium pit mine (PM pond) in the present study, and in the uranium mine (M pond) located in Portugal in the study of Antunes et al. (2007).

		Present Study/uranium mine	**Antunes et al. (2007)/ uranium mine**	
		Brazil (PM pond)	**Portugal (M pond)**	
		Average	Spring	Autumn
48 h EC_{50} (% Effluent) and IC	*Ceriodaphnia silvestrii*	0.34<0.41<0.52	-	-
	Daphnia magna	4.77<5.41<6.18	80.5<83.6<87.2	26.75<35.8<50.78
	Daphnia longispina	-	41.6<49.3<60.2	16.95<20.5<24.25
Main Chemical Elements (mg L^{-1})	U	1.65	0.552	1.842
	Th	0.19	-	-
	Mn	29.17	7.016	11.86
	Al	54.1	0.495	9.07
	Zn	4.46	0.569	0.68
	Fe	7.25	1.692	10.65
	Sr	-	0.352	0.498
	Co	0.023	0.049	0.117
	Ni	0.05	0.116	0.193
	F^-	12.46	-	-
	pH	2.87	5.67	3.44

Figure 3.4.1 shows the results obtained in the 48 h acute toxicity tests for the samples from the two UTM/INB ponds, that is, the *in natura* effluent (PM) and the treated effluent (P41-S), as well as one point from the Antas reservoir, that is the reference site (CAB). According to the toxicity results, the survival of the control treatments was 100% for *C. silvestrii* and *D. magna* after 48 h. A statistical analysis of the mobility data from the acute toxicity bioassays with *C. silvestrii* and *D. magna* revealed acute toxicity effects in all the water samples from the P41-S and PM ponds for both daphnids tested ($P<0.05$). In addition, the results for acute toxicity registered in the samples from the P41-S pond, showed that the treated effluent did not conform to the standards for discharge into the environment, since the effluents from a polluting source must not cause or present the potential to cause toxic effects to aquatic organisms in freshwater bodies (Conama, 2011). On the other hand, the reference site (CAB point) did not present significant differences ($P>0.05$) in the toxicity results, when compared to the survival of the control treatments.

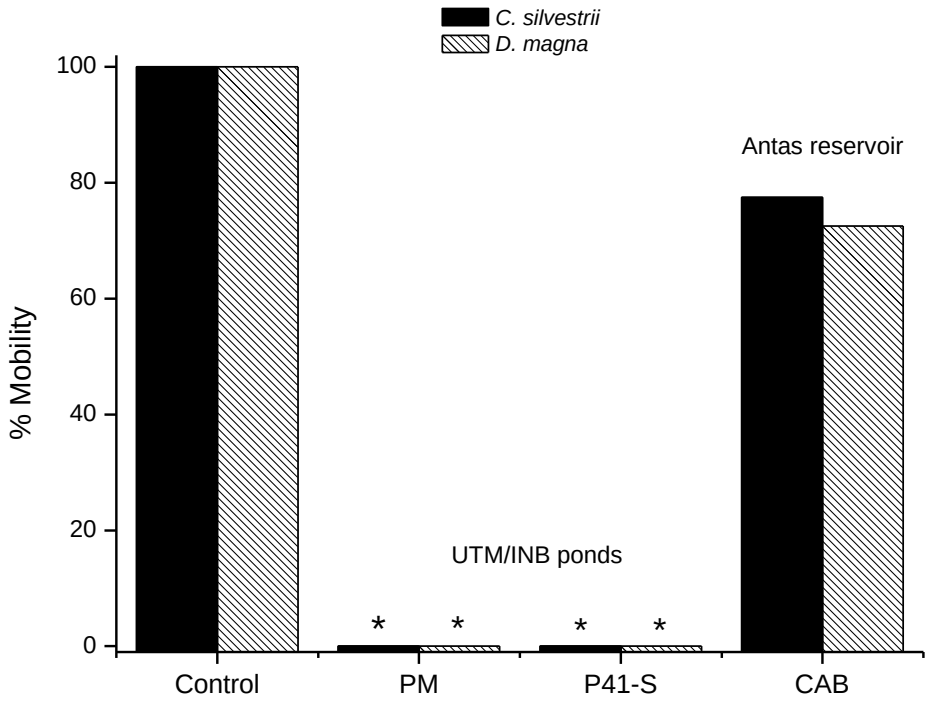

Figure 3.4.1 Percentage (%) of cladoceran mobility in the acute toxicity tests - * indicates a significant difference from the Control (Fisher's test, *P<0.05*).

3.4.4 Discussion

According to the statistical analyses, the *in natura* effluent samples from the PM pond, presented high concentrations of some chemical species, mainly: Al, Mn, Zn, U, Th, F^- and Fe, and also high values for hardness and electrical conductivity and acidic pH values, being considered acutely toxic at 100%, 50%, 25% and 12.5% for all the organisms, as can be seen from the results for toxicity (0% mobility in all samples tested) (Figure 3.4.1).

Due to the very high toxicity potential of this effluent, a set of appropriate dilutions was used to obtain the EC_{50} values in the best confidence interval, as follows: 0.34<0.41%<0.52 for *C. silvestrii* and 4.77<5.41%<6.18 for *D magna*. In the PM pond, the low pH values associated with high concentrations of many chemical elements in the water samples from the *in natura* effluent, throughout the whole period studied, produced a significant toxic effect, which could only be decreased by increasing the proportion of dilution water in a sequential set of dilutions, indicating that this effluent was acutely toxic for both species due to the low EC_{50} values, registered as percentages. Artal et al. (2013) also assessed the effluent from this same uranium mine in Poços de Caldas (Minas Gerais, Brazil),

and verified that the *in natura* effluent samples (at concentrations of 0.1, 1.0, 10 and 100%) were acutely toxic to *Daphnia similis*, with the EC_{50} values ranging from <0.9% to 2.41% without pH adjustment, but up to 38% after pH adjustment (to pH 7.0 ± 0.5) at the same concentrations. Comparing the acute toxicity results obtained for the *in natura* effluent samples in present study with *D. magna* (4.77<5.41%<6.18) with the toxicity results reported for *D. similis* (<0.9% - 2.41%) in the study carried out by Artal et al. (2013), it can be seen that *D. magna* was as sensitive as *D. similis*, since that this effluent was acutely toxic for both species.

In a study carried out in an abandoned uranium mine in Cunha Baixa, Mangualde (Portugal), with high concentrations of some metals (i.e. Al, Mn Fe, Co and U) and low pH values (3.44 and 5.67 in water samples collected in the autumn and spring), Antunes et al. (2007a) verified that the significant increase in the acute toxicity of the effluent for *D. magna* was related to the more acidic pH values, that is, the EC_{50} values ranged from 35.8% to 83.6%, when the pH values were 3.44 and 5.67, respectively. Comparing the toxicity results obtained by Antunes et al. (2007a) for *D. magna* in the spring and autumn, ranging from 35.8% to 83.6%, with the average EC_{50} value obtained in the present study for the same species, of 5.41%, it can be seen that the toxicity registered in the present study was much more pronounced than that registered by Antunes et al. Moreover, in the present study the more acidic pH values could also have favored greater solubility of the metal mixture (Mn, Al, Th, Zn and F^-) in the PM pond, which occurred in higher concentrations or were not analyzed, such as Th and F^-, as compared to the values obtained by Antunes et al. (2007a). Thus in the present study, the toxicity potential of the *in natura* effluent from the PM pond for the daphnids *C. silvestrii* and *D. magna* was more pronounced, as can be seen from the EC_{50} values registered (Table 3.4.2).

Furthermore, in the present study the bioassays showed that *C. silvestrii* was more sensitive to the *in natura* effluent from the PM pond for all the samples, according to the CE_{50} values that ranged from 0.16% to 0.69%. According to Harmon et al. (2003) the use of indigenous species may reflect a more realistic test of the locality, since these species are representative of the local flora. Thus, in the present study the use of indigenous species such as *Ceriodaphnia silvestrii*, could contribute with the generation of more relevant ecotoxicological data, reflecting the possible consequences of the presence and/or launching of toxic agents in relation to the main groups of organisms existing in the environment under observation (Freitas and Rocha 2010; Souza et al. 2014). In agreement with this assumption, Antunes et al. (2007a) also pointed out that the use of native/indigenous species, such as

Daphnia longispina, common in Portuguese freshwaters, might provide more ecologically relevant information on the toxicity potential, since this specie was shown to be more sensitive than *Daphnia magna* according to the EC_{50} values recorded for the effluent samples collected in the autumn and spring, respectively of 20.5% and 49.3% for *D. longispina* and 35.8% and 83.6% for *D. magna* (Table 3.4.2).

In addition, the total uranium concentration recorded in the PM pond (from 1.54 to 1.76 mg L^{-1} U) was about 23 to 3 times higher than the EC_{50} values registered for *C. silvestrii* at pH 7.5 and *D. magna* at pH 7.0, which were 0.07 mg L^{-1} U and 0.58 mg L^{-1} U, respectively (Ferrari et al., unpublished data). Moreover the uranium concentrations found in the water samples from the PM and P41-S ponds, were above the limits for uranium chemical toxicity for aquatic invertebrates as reviewed by Sheppard et al. (2005), who proposed a predicted no-effect concentration (PNEC) of 0.005 mg L^{-1} U as protective for most aquatic organisms. In addition, the Mn concentrations recorded in the water samples from the PM pond (from 25 to 34.6 mg L^{-1} Mn) were also higher than the Mn 48 h EC_{50} value for *C. silvestrii*, which was 5.93 mg L^{-1} (Ferrari et al., unpublished data). Thus the EC_{50} results registered for U and Mn indicated a high toxicity potential to daphnid species of the *in natura* effluent in the PM pond.

Antunes et al. (2007) also analyzed the elutriate samples from the sediment of the same uranium mine in Cunha Baixa, Manguale (Portugal) in three ponds, that is, in the mine pit pond, reference pond and treatment pond, and found that the reference and treatment ponds were not toxic to the daphnids (*D. longispina* and *D. magna*). However acute toxicity was observed in the elutriate sediments from the mine pit pond in the spring, which was probably related to the low pH value (~5), which may have contributed to mobilization of particle bound contaminants in the sediment, which thus became bioavailable to the aqueous phase. In the present study, the more acidic pH values recorded in the PM pond throughout the whole study period may also have promoted a greater bioavailability of the contaminants associated with the sediment from this site, favoring their remobilization to the water column such that they became bioavailable and hence contributed to the high toxicity potential of this effluent, as observed in the EC_{50} values registered.

In another study carried out in the same uranium pit mine (PM pond), Ferrari et al. (2015) concluded that potential stressors (i.e. moderately acidic pH and high concentrations of stable and radioactive contaminants), probably influenced the occurrence of the cladoceran species at this site, since the rotifers were the most representative organisms throughout the study, followed by the occasional presence of *Bosminopsis deitersi* and *Bosmina* sp. (smaller cladocerans). As observed by Ferrari (2015) in the uranium pit mine (PM pond) the relative

importance and dominance of small rotifers over crustacean zooplankton was also detected in other studies in acidic pit lakes (Belyaeva and Deneke 2013; Deneke 2000; Wollmann et al. 2000). In the present study, the *in natura* effluent samples from the PM pond were acutely toxic to the larger cladocerans, represented by *C. silvestrii* and *D. magna*. The extinction of the larger-bodied zooplankton in the study carried out by Ferrari et al. (2015), and the acute toxicity results registered in the present study for the larger cladocerans, were probably related to the acid stress caused by the H^+ concentration and the toxic stress of the metal mixture, which can impair the normal process of osmoregulation in acid-sensitive zooplankton (Havens 1993). In a study carried out at the Antas reservoir, Ferrari (2010) verified differences in the composition of the zooplankton community in this reservoir, where the small rotifers dominated samples taken at the P41-E point (where treated effluent proceeding from P41-S is discharged into the environment) over zooplankton crustaceans. On the other hand, the crustaceans (larger-bodied zooplankton) dominated at the reference site (CAB) and downstream from P41-E, that is, at the P14 point. This dominance of the Rotifera group observed in the P41-E and PM pond water samples in the studies carried out by Ferrari (2010) and Ferrari et al. (2015), may be due to the fact that the rotifers are considered to be the most tolerant taxa to variations in pH, and are also the least sensitive group to metal concentrations (Jak et al. 1996).

It was also observed that the treated effluent samples from the P41-S pond also presented high concentrations of some chemical species (i.e. F^- and SO_4^{2-}, and high electrical conductivity and hardness), with the ion F^- presenting concentrations above the limits established by current Brazilian legislation, that is, 10 mg L^{-1} for the discharge of treated effluents (Conama 2011). In agreement with the results obtained for the PM pond, samples from the P41-S pond were also considered to be acutely toxic for the organisms (0% mobility in all samples). On the other hand, according to the statistical analysis of the samples from the reference site, CAB point, these presented lower metal concentrations than those found in the P41-S and PM ponds, with the exception of Al(d), Cu(d), Fe(d) and Mn, which were slightly above the limits established by current Brazilian legislation (Conama 2005). According to the toxicity results the CAB point presented acute toxicity levels that were not different ($P>0.05$) from those of the control treatment (Figure 3.4.1).

Antunes et al. (2007) verified that the chemical treatment carried out on the acid effluent from an abandoned uranium mine pond (Cunha Baixa uranium mine), presented positive outcomes, since no toxicity was registered for *D. longispina* and *D. magna* in the treatment pond at this site. On the other hand, in the present study the toxicity results as well

as the physicochemical data, indicated that the treatment of the *in natura* effluent from the PM pond carried out by the mining company (UTM/INB) was improper and inefficient, since the treated effluent samples from the P41-S pond were as toxic as the *in natura* effluent samples from the PM pond for both daphnids. These results agreed with those of Artal et al. (2013) who also analyzed the uranium mine effluent at Poços de Caldas and reported that the physical chemical treatment applied by the mining company to this effluent was not efficient in removing the chronic toxicity for *Ceriodaphnia dubia*. Moreover, Rodgher et al. (2013) detected seasonal variations in the levels of some chemical elements such as fluoride, sulfate, manganese and uranium in a study also carried out at the Antas reservoir, and reported that these variations were probably a result of the improper treatment of the uranium mine effluent by UTM/INB, which was then discharged into the reservoir. Thus it is recommended that the discharge of treated effluent into this reservoir be carefully monitored in relation to the chemical and ecotoxicological water quality.

It is worth mentioning that the results registered for some chemical species in the P41-S pond were considered high (i.e. U, Zn, Mn, Al, SO_4^{2-} and F^-), since the effluent from this pond will be discharged into the Antas reservoir at the P41-E point, where the standards demanded for water chemical quality are more restricted according to the Conama Resolution (2005), than those of the legislation for the chemical water quality designated for treated effluent, which is the case of the P41-S pond (Conama 2011). Thus it is suggested that the treated effluent discharged into the Antas reservoir undergo dilution in this aquatic system. It should also be considered that the discharge of treated effluents that do not conform to the discharge standards determined by Brazilian legislation (Conama, 2011) into the natural waters and rivers downstream, may also affect the survival and composition of the local flora, due to possible short and long-term toxic effects.

3.4.5 Conclusions

Based on the acute toxicity results of the present study, as well as the physicochemical data, the treatment of the *in natura* effluent of the PM pond carried out by the mining company (UTM/INB) was found to be improper and inefficient, since the treated effluent in the P41-S pond was as toxic as the *in natura* effluent samples of in the PM pond for both daphnids. Thus, the authors suggest that ecotoxicological and physicochemical monitoring be carried out continuously in this reservoir in order to predict the impacts of the discharge of the

uranium mine effluents on the chemical water quality and on the local freshwater fauna of the system.

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4 GENERAL CONCLUSIONS

✓The cladocerans *C. silvestrii* and *D. magna* cultivated in the Ecotoxicology Laboratory of LAPOC/CNEN showed reliable conditions to performing acute toxicity bioassays, since that the cultivation conditions agreed with those of the international and national norms;

✓Samples of the *in natura* and treated effluents presented seasonally higher concentrations of chemical species in relation to the reference point at the Antas reservoir, and were considered acutely toxic to the cladoceran *Ceriodaphnia silvestrii and D. magna*;

✓Seasonally, a clearly protective effect to the acute toxicity of the chemical mixture in water from the Antas reservoir was provided to the cladocerans *C. silvestrii* and *D. magna* by very high hardness values;

✓Mn values registered in the literature for water samples from the Antas reservoir revealed toxicity potential for the indigenous specie *C. silvestrii*, as observed in the EC_{50} value registered that was of 5.92 mg L^{-1}Mn;

✓The bioassays showed that *C. silvestrii* (EC_{50} = 0.07 mg L-1 U) was more sensitive to uranium metal than *D. magna* (EC_{50} = 0.56 e 2.4 mg L^{-1} U at pH 7 and 8, respectively), a fact that indicated the importance of using indigenous species, since that it represent the local flora.

✓When the pH value was decreased from 8 to 7 the speciation of the metal uranium was shown to change, with greater concentrations of the species UO_2^{2+} and UO_2OH^{+}, considered potentially toxic to the biota, being registered at pH 7;

✓The acute toxicity results, indicated that the treatment of the *in natura* effluent from the PM pond carried out by the mining company (UTM/INB) was improper and inefficient, since the treated effluent samples from the P41-S pond were as toxic as the *in natura* effluent samples from the PM pond for both daphnids.

- ✓Samples of the *in natura* effluent from the pit mine pond presented very high toxicity potential for the *C. silvestrii* and *D. magna*, according to the extremely low values EC_{50} registered that were: 0.34<0.41%<0.52 and 4.77<5.41%<6.18, respectively;

APPENDIXES A

Chemical composition of the culture medium for the Cladoceran species *Ceriodaphnia silvestrii.*

Solutions	Reagent	Amount
1	$CaSO_4.2H_2O$	1500
2	KCl	200
	$NaHCO_3$	4800
	$MgSO_4.\ 7H_2O$	6100

Concentrations are given in mg/L

ABNT (2010)

APPENDIXES B

Chemical composition of the culture medium for the Cladoceran species *Daphnia magna*; Fonte: Elendt and Bias, 1990.

Medium		ISO	M 4
	Trace nutrients[a]		
B	(H_3BO_4)	–	0.5000
Fe	($FeSO_4 * 7 H_2O$)[b]	–	0.2000
Mn	($MnCl_2 * 4 H_2O$)	–	0.1000
Li	(LiCl)	–	0.0500
Rb	(RbCl)	–	0.0500
Sr	($SrCl_2 * 6 H_2O$)	–	0.0500
Mo	($Na_2MoO_4 * 2 H_2O$)	–	0.0250
Br	(NaBr)	–	0.0125
Cu	($CuCl_2 * 2 H_2O$)	–	0.0063
Zn	($ZnCl_2$)	–	0.0063
Co	($CoCl_2 * 6 H_2O$)	–	0.0025
J	(KJ)	–	0.0025
Se	(Na_2SeO_3)	–	0.0010
V	(NH_4VO_3)	–	0.0003
	$EDTA * 2 H_2O$[b]	–	2.5000
	Macro nutrients		
$CaCl_2 * 2 H_2O$		293.80	293.80
$MgSO_4 * 7 H_2O$		123.30	123.30
$NaHCO_3$		64.80	64.80
KCl		5.80	5.80
$Na_2SiO_3 * 9 H_2O$		–	10.00
$NaNO_3$		–	0.27
KH_2PO_4		–	0.14
K_2HPO_4		–	0.18
	Vitamins		
Thiamine[c]		–	75.0
B_{12}[c]		–	1.0
Biotin[c]		–	0.75

Concentrations are given in (mg/l), unless otherwise stated

[a] Concentrations are given for the target element (source compounds are given in brackets)

[b] Stock solutions of $FeSO_4 * 7 H_2O$ and $Na_2EDTA * 2 H_2O$ are poured together and autoclaved immediately

[c] (µg/l)

APPENDIXES C

Chemical composition of the culture medium CHU for the alga *Raphidocelis subcapitata*.

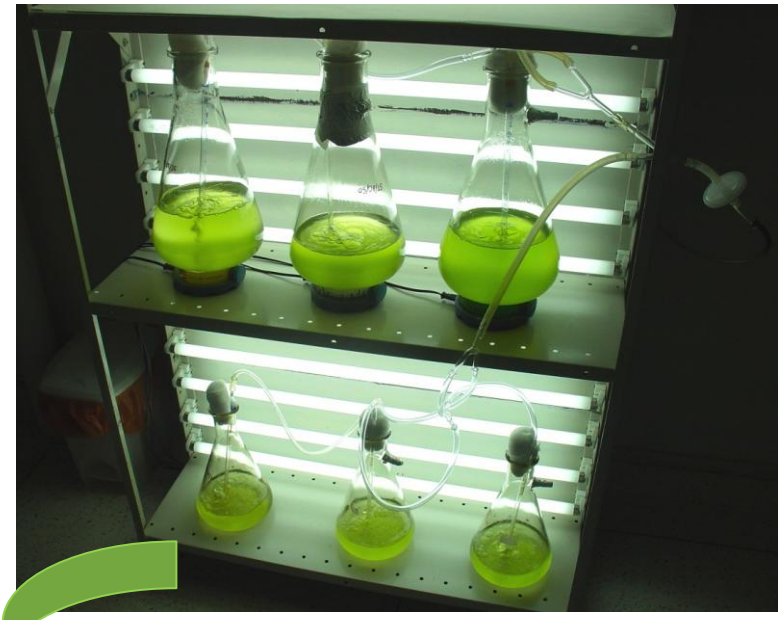
CHU Medium	
Nutrients	
$Ca(NO_3)_2$	0.430
K_2HPO_4	0.050
$MgSO_4.7H_2O$	0.750
KCl	0.050
Na_2CO_3	0.200
$FeCl_3.6H_2O$	0.005

Concentrations are given in g/100mL
Müller (1972)

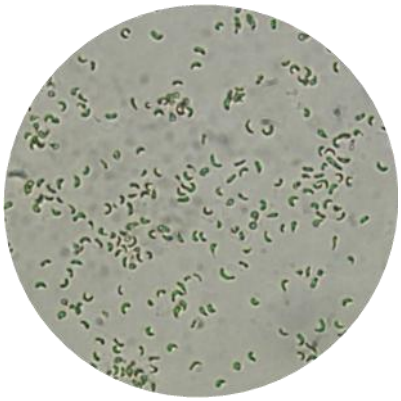
APPENDIXES D

A: Photo illustrating the cultivation of the alga *Raphidocelis subcapitata* under axenic conditions in the Ecotoxicology Laboratory of LAPOC/CNEN; **B:** Photo of the microscopic field showing the cultivation of spread cells of *R. subcapitata*;

A



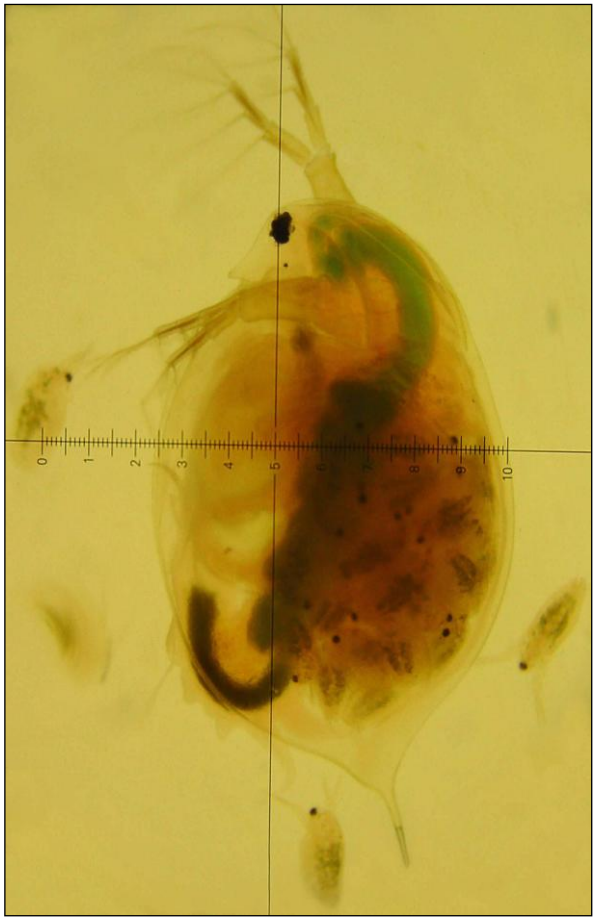
B



APPENDIXES E

A: Photo taken using a magnifying glass of an ovate adult female *Daphnia magna* (6.0 mm in length) cultivated in the Ecotoxicology Laboratory of LAPOC/CNEN; **B:** Photo taken using a magnifying glass of a male *Daphnia magna* (2.5 mm in length). Fotos: Ferrari, C. R.

A



B

APPENDIXES F

Photo carried out using the microscope (magnification x20) of an ovate adult female Ceriodaphnia silvestrii (1.1 mm in length) cultivated in the Ecotoxicology Laboratory of LAPOC/CNEN: Photo: Ferrari, C. R.

APPENDIXES G

Life cycle of *Daphnia magna*: Source: ISO:6341 (2012). Drawing: Lima, E. O. (2015).