UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Sorocaba

PROGRAMA DE PÓS-GRADUAÇÃO em
Ciências Ambientais

CLÁUDIA HITOMI WATANABE

FATE, BEHAVIOR AND ECOTOXICOLOGY OF SILVER NANOPARTICLES: INTERACTIONS WITH NATURAL ORGANIC MATTER IN AQUATIC SYSTEMS

Sorocaba
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Orientador: Prof. Dr. Marc F. Benedetti
Coorientador: Dr. Rute F. Domingos

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TÍTULO DA TESE: Fate, behavior and ecotoxicology of silver nanoparticles: interactions with natural organic matter in aquatic systems

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“Teach me good judgement and knowledge; For I have believed in thy commandments.”

(Psalms 119: 66 American Stadard Version)

Watanabe CH. **Fate, behavior and ecotoxicology of silver nanoparticles: interactions with natural organic matter in aquatic systems**. 2020. Thesis (Doctoral's degree in Environmental Sciences) - Campus de Sorocaba, UNESP – Sao Paulo State University, Sorocaba.

ABSTRACT

When introduced in environment, manufactured nanoparticles (NPs) can interact with biotic and abiotic molecules yielding a transformed NP usually coated with these molecules (natural coating). These different forms of the NPs should be considered as new materials because them distinct properties from the released NPs forms. In fact, these in situ transformations of the NPs have relevant impacts on their toxicological effects, having new or additional risks still not studied. This project aims to determine, understand and predict the impact of natural molecules in the transformations and bio-effects of Ag NPs, widely used as antibacterial agents. Despite the growing interest and use of these NPs, the gathered knowledge on their environmental consequences is still scarce, since the large majority of the studies do not consider the effect of the presence of natural coatings around the particles. In fact, most of the studies do not even determine the effects of the presence of manufactured coatings. Generally, NPs manufacturers add ionic or polymeric coatings to improve their mobility and stabilization in terms of size. Although the possible effects of these manufactured coatings in the NPs behavior there is not systematically studied about their impact. This project aims to overcome the current uncertainty about the environmental safety of manufactured coated NPs, and explore the impact of natural molecules on their environmental risk. The project objectives are: i) characterize the manufactured coated Ag NPs in absence and presence of natural molecules including Humic Substances (HS) and Extracellular Polymeric Substances (EPS), ii) determinate the biocompatible and bioadverse effects of the transformed NPs on the algae *Raphidocelis subcapitata* and the microcrustacean *Daphnia similis* and iii) evaluate the transfer of the transformed NPs in the food chain alga-microcrustacean. Thus, this project purposes to introduce a standardized and improved evaluation of the ecotoxicology in order to increase the competitiveness and safety of nanostructured products.

Key-words: nanoparticles; toxicity; humic substances; exopolysaccharides; silver.

Watanabe CH. **Destino, comportamento e ecotoxicologia de nanopartículas de prata: interações com matéria orgânica natural em sistemas aquáticos**. 2020. Tese (Doutorado em Ciências Ambientais) - Campus de Sorocaba, UNESP - Universidade Estadual Paulista, Sorocaba.

RESUMO

Quando introduzidas no ambiente, nanopartícula (NP) podem interagir com moléculas bióticas e abióticas produzindo uma NP transformada geralmente revestida com essas moléculas (revestimento natural). Essas diferentes formas de NPs devem ser consideradas como novos materiais, pois possuem propriedades distintas dos formulários liberados dos NPs. De fato, essas transformações in situ das NPs apresentam impactos relevantes em seus efeitos toxicológicos, tendo riscos novos ou adicionais ainda não estudados. Este projeto tem como objetivo determinar, entender e prever o impacto de moléculas naturais nas transformações e efeitos biológicos dos NPs de Ag, amplamente utilizados como agentes antibacterianos. Apesar do crescente interesse e uso desses NPs, o conhecimento acumulado sobre suas conseqüências ambientais ainda é escasso, uma vez que a grande maioria dos estudos não considera o efeito da presença de revestimentos naturais ao redor das partículas. De fato, a maioria dos estudos nem sequer determina os efeitos da presença de revestimentos fabricados. Geralmente, os fabricantes de NPs adicionam revestimentos iônicos ou poliméricos para melhorar sua mobilidade e estabilização em termos de tamanho. Embora os possíveis efeitos desses revestimentos fabricados no comportamento dos NPs não sejam sistematicamente estudados sobre seu impacto. Este projeto visa superar a atual incerteza sobre a segurança ambiental de NPs revestidos fabricados e explorar o impacto de moléculas naturais em seus riscos ambientais. Os objetivos do projeto são: i) caracterizar os NPs de Ag revestidos fabricados na ausência e presença de moléculas naturais, incluindo Substâncias Húmicas (HS) e Substâncias Poliméricas Extracelulares (EPS), ii) determinar os efeitos biocompatíveis e bioadversos dos NPs transformados nas algas *Raphidocelis subcapitata* e o microcrustáceo *Daphnia similis* e iii) avaliar a transferência dos NPs transformados na cadeia alimentar de microcrustáceos algas. Assim, este projeto tem como objetivo introduzir uma avaliação padronizada e aprimorada da ecotoxicologia, a fim de aumentar a competitividade e a segurança dos produtos nanoestruturados.

Palavras-chave: nanopartículas; toxicidade; substancias húmicas; exopolissacarídeos, prata.

Watanabe CH. **Destin, comportement et écotoxicologie des nanoparticules d'argent: interactions avec les matières organiques naturelles dans les systèmes aquatiques**. 2020. Thése (Doutorado em Ciências Ambientais) - Campus de Sorocaba, UNESP - Universidade Estadual Paulista, Sorocaba.

RÉSUMÉ

Lorsqu'elles sont introduites dans l'environnement, les nanoparticules manufacturées (NPs) peuvent interagir avec des molécules biotiques et abiotiques, produisant des NPs transformées, recouvertes par ces molécules, colloïdes naturels. Ces différentes formes de NP doivent être considérées comme de nouveaux matériaux, car elles ont des propriétés différentes des NPs sources libérées dans le milieu naturel. En fait, ces transformations in situ des NPs ont des impacts significatifs sur leurs effets toxicologiques, avec des risques nouveaux ou supplémentaires peu étudiés. Ce projet vise à déterminer, comprendre et prévoir l'impact des molécules naturelles sur les transformations et les effets biologiques des NPs d'Argent, largement utilisés en tant qu'agents antibactériens. Malgré l'intérêt croissant et l'utilisation de ces NPs, les connaissances recueillies sur leurs conséquences pour l'environnement sont encore rares, car la grande majorité des études ne prend pas en compte l'effet de la présence d'enrobages d'origine naturel autour de ces particules. En fait, la plupart des études ne déterminent même pas les effets de la présence de enrobages manufacturés (par opposition aux enrobages naturels) accompagnant les NPs. Généralement, les fabricants de NP ajoutent des enrobages ioniques ou polymériques pour améliorer la mobilité et la stabilité des NPs et leur impact n'est pas systématiquement étudié. Ce projet vise à surmonter les incertitudes actuelles quant à la sécurité environnementale des nanoparticules enrobées et à explorer l'impact des molécules naturelles sur leur risque environnemental. Les objectifs du projet sont les suivants: i) caractériser les NP Ag avec de enrobages manufacturés en l'absence et en présence de molécules naturelles, y compris les substances humiques (HS) et les substances polymériques extracellulaires (EPS), ii) déterminer les effets positifs et/ou néfastes des NPs ainsi transformées sur l'algue Raphidocelis subcapitata et le micro-crustacé Daphnia similis et iii) évaluer le transfert des NPs transformés dans une chaîne alimentaire modèle simplifiée: algue-micro-crustacé. Ce projet vise donc à introduire une évaluation standardisée et améliorée de l'écotoxicologie afin d'accroître la compétitivité et la sécurité des produits nanostructurés.

Mots-clés: nanoparticules; toxicité; substances humiques; exopolysaccharides; argent.

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I. INTRODUCTION

The current expansion of nanoscience and nanotechnology and the massive use of nanoparticles (NPs) that have at least one dimension in the nanoscale (1-100 nm) in commercial products reflect in the increase of these materials in the biosphere. Generally, NPs manufacturers add ionic or polymeric coatings to improve their mobility and stabilization in terms of size. Despite the possible effects of these manufactured coatings on the NPs behavior there is not systematically studied. Due to the importance of these coatings and the large impact on its reactivity, biophysical and chemical interactions with the surrounding matrices, commercially relevant silver nanoparticles (AgNP) coated will be evaluated. The use of these coatings allows covering a variety of surface modifications of NPs, engineered with a propensity to solubilize and others much more stable in terms of size and solubility.

When introduced in the environment, these engineered nanomaterials (ENM) can interact with biotic and abiotic molecules yielding a transformed nanoparticle usually coated with these molecules (natural coating, or ecocorone). These different forms of the NPs should be considered as new materials because they have distinct properties from the initial released NPs forms. In fact, these *in situ* transformations of the NPs have relevant impacts on their toxicological effects, having new or additional risks still not studied. Despite the growing interest and the use intense of these NPs, it is reasonable to investigate the environmental consequences of these material, considering the effect of the presence of natural coatings around the particles as reported in literature (Wagner, 2014); (Grillo *et al.*, 2015); (Mensch *et al.*, 2017).

Furthermore, considering the bio distribution and toxicity of manufactured NPs dependent of the interactions with natural organic matter, the present work aims to determine, understand and predict the impact of natural components in the interactions of NPs manufactured in environmental and biological matrices. This project will address key issues that underlie the effort to understand how NPs behave in natural systems and how they can affect the environment, including the toxic potential accodring as represented in the Figure 1

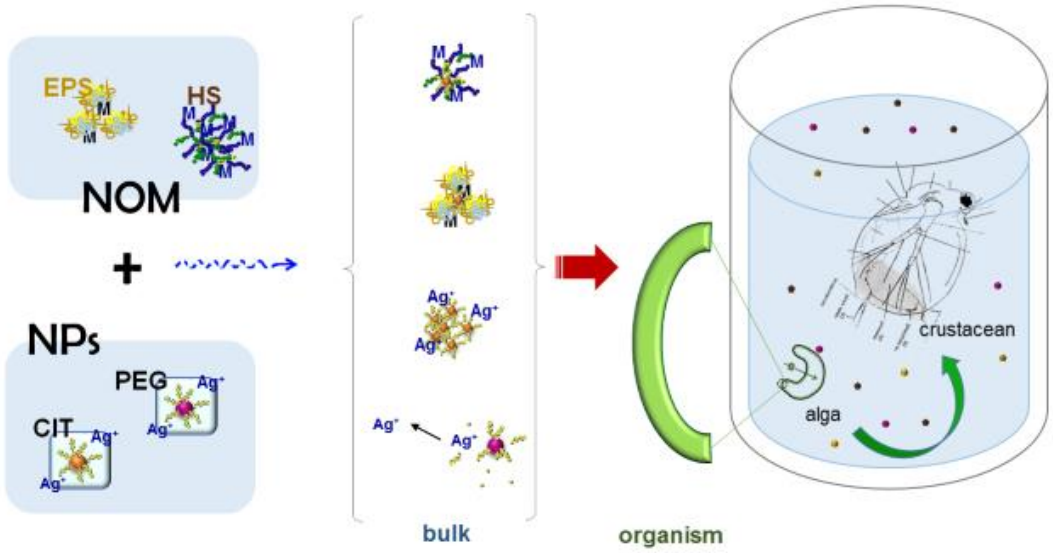


Figure 1 - Graphic abstract of the thesis considering the fate, internalization and behavior of silver nanoparticles in a food chain.

Then, the image represent some issues about the fate of AgNPs in the aquatic systems: what it will be the fate of AgCit and AgPEG with the same size, 10 nm, in the presence of natural organic? With the study of the speciation of the NPs it will be possible to link the resulting NPs forms with the internalization by the organisms present in those systems? Finally, how those transformed AgNPs will behave in a food chain?

II. Aims of thesis

In this context, the present work intents to contribute with the current uncertainty about the environmental safety of manufactured coated NPs, and explore the impact of natural molecules on their environmental risk. The work objectives are: i) characterize the manufactured citrate (Cit) and polyethilene glycol (PEG) caspped silver nanoparticles in absence and presence of natural molecules including Aquatic Humic Substances (AHS) and Extracellular Polymeric Substances (EPS), ii) determine the toxic effect of the transformed NPs on the algae *Raphidocelis subcapitata* and the microcrustacean *Daphnia similis*, and iii) evaluate the transfer of the transformed NP in the food chain alga-microcrustacean. Thus, the present work purposes to introduce

a standardized and improved ecotoxicology evaluation in order to increase the competitiveness and safety of nanostructured products.

III. Manuscript presentation

The thesis structure was organized into 6 sections. The first one addresses the state-of-art and main concepts related to the proposed theme subdivided into contextualization, general and specific objectives, as well as the organizational structure of the work (Figure 2). The middle section is divided into 4 chapters directly related to the development of the thesis involving the characterization and interactions of organic matter with AgNPs and the toxicity related. Finally, the sixth section presents the final considerations and future perspectives related to the main finding of the work associated to the fate of nanoparticles in the aquatic systems.

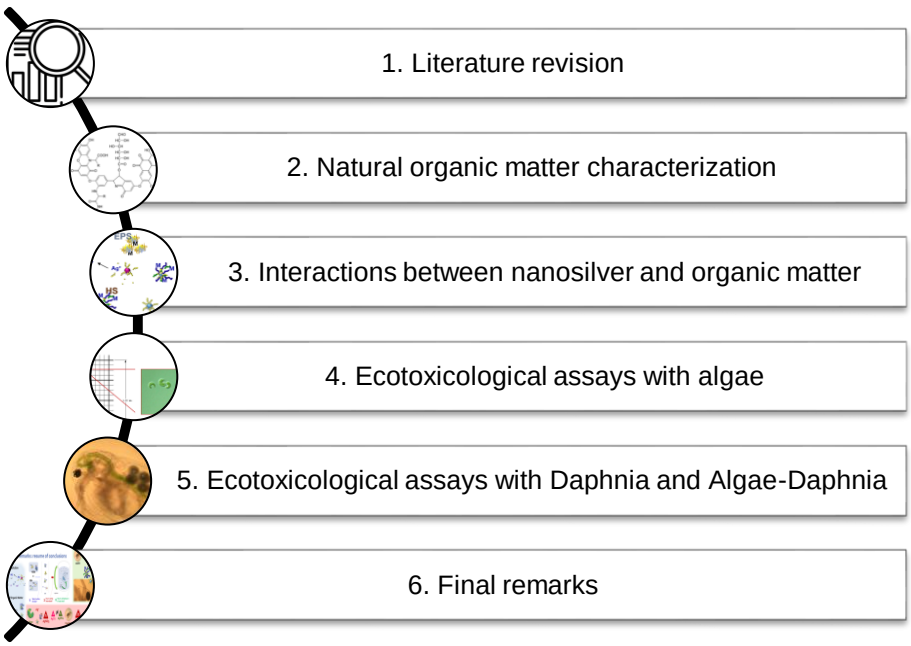


Figure 2 - Organizational structure of the thesis.

Chapter 1 presents an overview and contextualization of nanotechnology which addresses the main concepts, characteristics and applications related. State-of-the-art information allows guidance as well as contextualizing for the experimental stage of research that sought to achieve the proposed work, reported in the chapters 2 to 5.

The Chapter 2 is related to the sampling stage, extraction and characterization of natural organic matter to be studied: aquatic humic substances from river and exopolysaccharides produced from the microalga *Raphidocelis subcapitata*.

Chapter 3 describes the study of interactions between silver nanoparticles composed of different capping agents (citrate and PEG) with natural organic matter on dissolution and charge density in algal and daphnia culture medium.

The subsequent chapters present the toxicity response due to those interactions organic matter and nanomaterials. Chapter 4 discusses the effects of natural organic matter, represented by AHS, on the evaluation of the toxicity of *Raphidocelis subcapitata* microalgae and the influence of the presence of organic matter on bioaccumulation by comparing the effects of silver ions and nanoparticles. Still focusing on the ecotoxicological approach, Chapter 5 presents an evaluation of the toxicity of nanomaterials in the presence of organic matter and the effects of possible transformations of this emerging material under freshwater microcrustacean *Daphnia similis*. From the characterization results and ecotoxicological assays mentioned in the previous sections, Chapter 6 exposes a resume of the final remarks obtained in this work and present future perspectives related to nanomaterials studies. The bibliographical references are presented at the end of the thesis.

CHAPTER 1: LITERATURE REVISION

The fate and transport of pollutants in the hydrosphere are hardly to be predicted due to their distribution in the aqueous and/or solid phase and aquatic life. The chemical fate and transport in water are dependent of chemical reactions that result in dissolution, precipitation, hydrolysis, complexation, oxidation-reduction, and photochemical reactions. These processes are very much influenced by chemical phenomena such as bioaccumulation and magnification in food chains and biodegradation (Manahan, 2005).

As one, dynamic systems, they have variable inputs and outputs. Compared to the carefully controlled conditions of the laboratory, it is much more difficult to describe chemical phenomena in natural water systems. As mentioned, such systems are very complex and a description of their chemistry must take many variables into consideration. In addition to water, these systems contain mineral phases, gas phases, and organisms. To understand water pollution, it is first necessary to have an appreciation of chemical phenomena that occur in water (Manahan, 2005)

1.1 Natural organic matter

1.1.1 Aquatic humic substances

The organic matter present in soils, peat and sediments consists in a mixture of substances in various stages of decomposition. Originated by the biological and enzymatic decomposition of plant and animal residues generally present in the soil humic substances (HS) is the main constituent of natural organic matter (NOM), distributed globally in terrestrial and aquatic environments (Stevenson, 1994). It is estimated that half of the dissolved organic carbon (DOC) in surface and ocean waters is HS-type refractory organic matter known as aquatic humic substances (AHS) (Rocha and Rosa, 2003). They can be divided into three components according to the solubility: humic acids (portion not soluble in water when at pH below 2), fulvic acids (soluble portion in any pH condition) and humin (non-water soluble portion independent of pH) (Maccarthy, 1990).

In general, HS present characteristics such as: binding to numerous organic substances, formation of complexes with metal ions and colloids, influence on the brown or bronze coloration of water when in high concentrations (acting on the absorption of light and influencing the processes of photosynthesis) and resistance to biological degradation (Santos *et al.*, 2011).

1.1.2 Extracellular Polymeric Substances

Extracellular polymeric substances (EPS) are matrix is formed by different biochemicals secreted by microbes, release of cellular material/products by cell lysis or organic matter in the medium resulted from physiological activities of microorganisms in natural environments. Exudates from algae, mainly consisting of organic acids of low molecular weight and extracellular polysaccharide, play a significant role in geochemistry processes including reduction of metals, the rate and extent of dissolution of minerals and can thus also affect the fate of NPs in the environment, mainly metal NPs as in the case of those to be studied in this project (Seders and Fein, 2011). The literature suggests that the observed effects are likely to be due to the presence in the exudates of functional groups of organic acid-active protons, which will interact with metals and mineral surfaces. Tonietto et al. (2014) have investigated the ability of exudates have binding metals in the ionic form as Copper, Zinc, Cadmium and Lead. Despite this probable evidence in related to the impact of the fate of NPs, there are few studies involving the effect of EPS on the physicochemical properties of NPs (Zhou *et al.*, 2016).

1.2 Nanoparticles

1.2.1 Concept

According to European Union (2011), a nanomaterial means:

> “A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the

number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.

In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.

By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials".

In general, nanoparticles (NPs) are defined as particles that have at least one dimension in the nanoscale (1-100 nm). NPs are not only small crystals but an intermediate state between molecular and bulk materials exhibiting magnetic, electrical, optical, mechanical, unique chemical and structural; a consequence of the high ratio between surface area and volume, which results in a high percentage of surface atoms. These unique properties reflect in the increase of these materials in the biosphere due to the application of NPs in a wide variety of areas, including medical, remediation, environmental, computer, textile and energy (Ju-Nam and Lead, 2008). These emerging materials are unique and xenobiotics, and therefore of great focus on current scientific literature, including the determination of their fate, transport and toxicity.

1.2.2 Origin

NPs occur both in nature and as a result of anthropogenic sources, including industrial and combustion processes, and as a result of the emerging nanotechnologies. These materials can be categorized into three types according to their source (Farre *et al.*, 2011; Epa, 2017):

1) Natural ultrafine particles include: combustion products, viruses and sea spray, volcanic eruption, hydrothermal vent systems, physico-chemical wearing of rocks and dust volatilization, biological processes, ultraviolet (UV) degradation from aquatic systems, nucleation processes, metal-sulfide nanoclusters, hydrous iron, manganese oxide, nanominerals (e.g., ferrihydrite), natural organic-material aggregates, biogenic origin (e.g., uraninite).

2) Incidental Nanomaterials are generated by anthropogenic processes and include combustion process (diesel exhaust), welding fumes and industrial emissions (effluents), sorption and transport from aquatic systems, deposition from atmosphere.
3) Engineered NMs are designed with very specific properties and are made through chemical and/or physical processes. Main sources of nanomaterials in the environment, nanotechnology-production processes, spillage from nanotechnology.

1.2.3 Engineered Nanomaterials: Silver nanoparticles

The metallic AgNP are currently used in a large range of products due to the well-known antimicrobial and antifungal activity of the Ag^{+} ion and was estimated having the highest annual production volume (55 ton $year^{-1}$) (Piccinno *et al.*, 2012). Uncountable are the applications related to nanoparticles and then several different industrial sectors are included in the AgNPs application: disinfection of air and water, textiles, biomedical devices, clothing, cosmetics, paints, food packaging (Panyala *et al.*, 2008; Fabrega *et al.*, 2011).

Engineered Nanoparticles (ENP) are covered with manufactured coatings, whose function is to improve the mobility of the particles protecting them from unwanted reactions and preventing homoaggregation by charge repulsion and/or steric effects. These coatings are actually a barrier between the NPs and the matrix where they are dispersed can be from simple binders such as citrate (Cit) low polymers and high molecular weight (usually charged polymers such poly acrylic acid (PAA), or neutral such as polyethylene glycol (PEG), resulting in a significant difference in the changes that NPs can undergo, and therefore the target and toxicity.

Citrate coatings are generally less stable compared with polymer in general, resulting in greater solubility and aggregation of metal NPs. However, the use of a charged polymeric coating can lead to higher solubility of the metal NP under physicochemical conditions that favor metal complexation by its own functional groups of the polymer. In these cases, solubilization of the obtained metal core NP will occur until equilibrium is reached, not meaning that the released metal concentration equal to the concentration of free metal in solution as part will become complexed by the

functional groups (Domingos, Franco*, et al.*, 2013). A neutral polymeric coating avoids metal complexation resulting in the absence of NP solubilization, in addition to stabilizing the particles in terms of size more effectively.

1.2.4 Nanoparticle and transformation in the environment

Indeed, it has been used quite a significant effort from the academic and industrial community around the world converge studies in a solid framework for the assessment of nano safety and mitigate the risks associated with NPs (Klaine *et al.*, 2008). In this context, numerous manuals, guidelines and reports are available to the physical, chemical and biological of NPs. However, most of these studies do not consider the effect of the presence of coatings around these particles, whether they are manufactured and/or natural. The first result from the addition of stabilizers by the manufacturers in order to improve the mobility of the particles and stabilization in terms of size and the latter resulting in interactions between environmental and biological constituents. Thus, the highly dynamic behavior of NPs in environmental systems results in a set of physical and chemical transformations (Figure 1. 1) including dissolution and / or changes in size and shape of NPs (Ju-Nam and Lead, 2008). Although these transformations significantly influence the transport and toxic properties of NPs, available knowledge is scarce, vague and lacking in standardization.

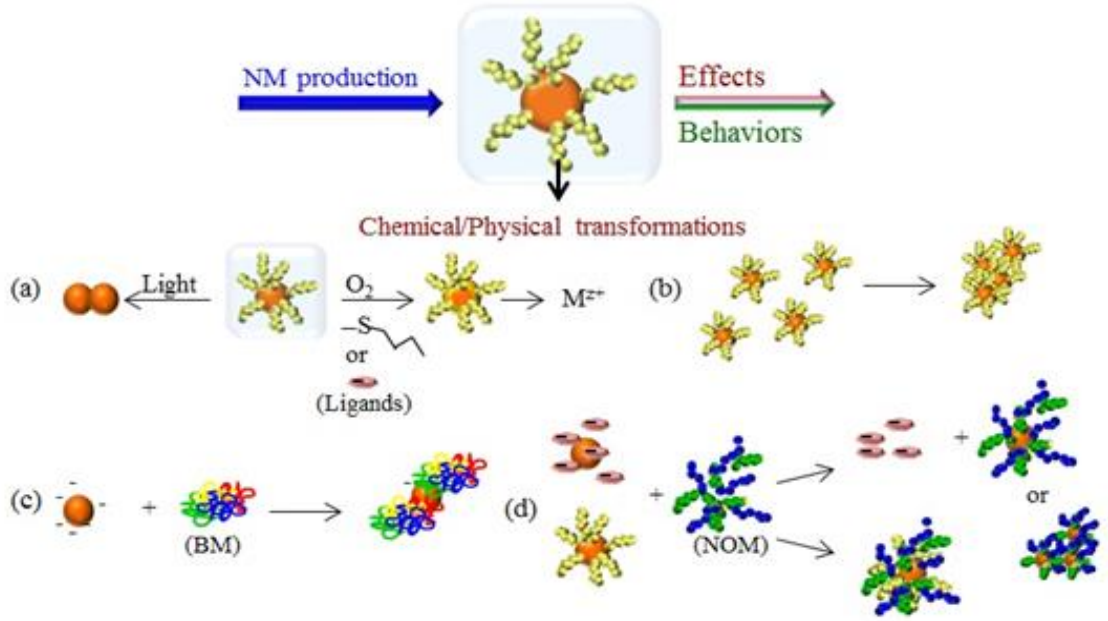


Figure 1. 1 - Representation of physico-chemical transformations of metal NPs. (a) photodegradation of the manufactured coatings which may result in aggregation, oxidation, thiol bonds and other bonds which may promote dissolution; (b) homoaggregation and heterocoagulation, including adsorption of (c) biomolcules (BM) and (d) natural organic matter (NOM). Source: Domingos and Pinheiro (2014).

A large number of NPs may be composed or contain components able to undergo (i) reduction and/or oxidation (Derfus *et al.*, 2004; Lok *et al.*, 2007; Shi *et al.*, 2011), (ii) photoreaction (Zhang *et al.*, 2007), iii) dissolution and sulfidation (Heinlaan *et al.*, 2008; Li *et al.*, 2011; Domingos, Rafiei*, et al.*, 2013; Kaegi *et al.*, 2013) and (iv) homo (including the same NPs) and/or heteroaggregation (between NPs and another colloid) (Domingos *et al.*, 2009; Hotze *et al.*, 2010; Domingos, Rafiei*, et al.*, 2013).

In this context, the National Research Council of the United States (2012) proposed a new framework for research in environmental and human security: research should be focused on the understanding of "crucial elements in the interactions of NPs, i.e. the properties or physical, chemical and biological processes that are considered most relevant to the exposure assessment, hazard and therefore risks related to NPs". Despite the critical impact such as interactions and possible transformations of NPs may have, there is a lack of information about the types, speed and extent of changes expected for NPs in environmental and human systems, limiting the understanding of the impact of these changes on their fate, bioavailability and toxicity.

1.2.4.1 Nanomaterials and Natural Organic Matter interactions

One of the most critical transformations of NPs and in a way by now neglected is the adsorption of natural colloids, including natural organic matter. It is well known that the HS can modify the surface properties of natural aquatic colloids significantly influencing its transport, often due to increased electrostatic repulsion. Despite the importance of these substances limited data are currently available reporntng the aqueous stability resulted from NPs interactions with NOM (i.e. adsorption) in terms of environmental relevant conditions (Domingos *et al.*, 2009; Domingos *et al.*, 2010).

These natural entities bind to the surface of the NPs forming a coating around these affecting its physical-chemical and biological identity, and thus affecting its reactivity surface and interactions with cells and organisms. NOM can arrange to NPs electrostatic or steric stabilization, or result in flocculation (Baalousha *et al.*, 2008; Domingos *et al.*, 2009). The effects produced by NOM are complex and difficult to predict, however, it is extremely important to exploit these interactions as the NOM concentrations are orders of magnitude higher than these NPs concentrations and therefore quite likely to modify the properties substantially behavior of NPs(Labille and Brant, 2010).

In this context, factors to be considered in the risk assessment of NPs include (i) the shape of the NP that will be present in commercial products, including the presence of manufactured coatings, (ii) the potential that the material tends to be released in the surrounding environment, and (iii) the transformation of the material that may affect exposure. This complexity is not unlike the problems associated with exposure of dissolved metal in water; depending on the chemistry of the aquatic environment concerned, the metals can have different oxidation states or different degrees of bioavailability. These dynamic speciation studies of trace metals conducted in the past have resulted in theoretical models that can predict the bioavailability of metal species (Domingos *et al.*, 2015). As in the case of metals models, it is vital to predict the form of nanostructured materials, and therefore, their possible effects as natural compartments.

To achieve this essential objective is of most importance to rigorous quantification of the dynamic contribution of species containing NPs. In fact, most of the projects already financed on nano safety include assessments of toxicity with

various progress, but only in the presence of the original NPs (usually in the absence of surface modifications, or manufactured coatings). Therefore, for a relevant risk assessment it is necessary to understand the danger of such forms of NPs (speciation), in other words, after processing.

The absence of the study of dynamic speciation of NPs under different physicochemical conditions is in fact a gap in the studies of fate and toxicity found in the literature. By the time the shape of NP is established, many of the issues related to exposure can in fact be reduced to an accurate measurement of the amount of material. Thus, it is essential to understand the heteroaggregation, solubilization, mobility and effects of NPs transformed into a well-planned multidimensional array of environmentally representative materials and means freshwater. This approach will allow a breakthrough in the understanding of environmental and biological fate of NPs and will certainly have a considerable impact on regulatory guidelines.

1.2.4.2 Characterization of NPs transformation: emphasis for NOM

Several techniques are being used to characterize the fate of NMs in the environment. Grillo et al. (2015) have listed several methods used to characterize the interactions of NPs with organic matter. Among them, surface charge by electrophoretic mobility (EPM); dynamic light scattering (DLS), field flow fractionation (FFF), transmission electron microscopy (TEM), scanning electron microscopy (SEM), scannig tunneling microscopy (STM), atomic force microscopy (AFM), nanoparticle tracking analysis (NTA) for size and aggregation and Fourier Transformed Infrared (FT-IR), X-Ray for chemical bonding characterization. Field Flow Fractionation (FFF) also can provide information related to fractionation as well as ICP, HPSEC, HPLC. Fluorescence correlation spectroscopy (FCS) can report about size and Ultraviolet Visible (UV Vis) absorption spectroscopy about aggregation.

On the other hand, even with the large number of techniques available for quantification of NPs, most of them show high limits of detection (LODs), which makes them ineffective for an environmentally relevant analysis. For this reason, few analytical approaches are considered adequate for quantification of NPs in the environment (Farre *et al.*, 2011). Single-particle inductively coupled plasma mass spectrometry (sp-ICPMS) is considered a powerfull tecnhique for metallic ENP

detection and characterization (size distribution, particle number concentration, stability) due to its low concentration quantification in aquatic environment (Yang *et al.*, 2016).

1.3 Ecotoxicology of nanoparticles

While chemical analyzes identify and quantify substance concentrations, toxicity tests assess the effect of these substances on biological systems. Thus, chemical analyzes and toxicity tests complement each other. In the case of industrial effluent samples, because they have a complex chemical nature and numerous chemical substances, an individual characterization becomes economically unfeasible to detect all substances present (Costa *et al.*, 2008).

Toxic substances may be introduced into surface waters in the form of potentially toxic metals, inorganic reducing agents, organic compounds. Despite their dispersion and dilution in the aquatic environment, it is possible that metals and organic compounds accumulate in organisms living in these environments, directly interfering with the often unknown biological effects (Wetzel, 2001; Dodds, 2002). Some of the different responses between short- and long-term risks between organisms and toxic agents may, however, be specific to a particular toxic substance. Short-term responses allow the body to resist the toxic effect while long-term responses allow it to adapt to the presence of toxic substances. Investigation of sublethal parameters is very important and a wrong choice may result in the apparent absence of any exposure even when the organism has been exposed to a toxic agent. Moreover, understanding the physiology of organisms is essential in toxicology.

The purpose of these ecotoxicological assays is to assess toxicity through standardized methods to establish water quality criteria. The most common effect observed in a toxicity test is lethality. In a natural environment, contaminant concentrations are rarely so high as to cause direct lethality. On the other hand, sub lethal effects such as locomotion and reproduction difficulty, predation reduction or metabolic alterations are more commonly observed effects. There are numerous types of toxicity testing. The most commonly known types are acute and chronic toxicity tests for certain stages of life (Nikinmaa, 2014).

Another decision to be made to perform a response-based toxicity test is the choice of test organism. Ecotoxicological studies are performed using organisms from different food chain levels, especially those that are cultivable in the laboratory and which have standards for such assays, such as microalgae (*Chlorella vulgaris*, *Scenedesmus quadricauda*, *Scenedesmus subspicatus* and *Raphidocelis subcapitata*), diatoms (Navicula pelliculosa, Skeletonema costatum), or cyanobacteria (Anabaena flos-aquae, Synechococcus leopoldensis) bacteria (*Spirillum volutants*, *Pseudomonas fluorescens)*, cladoceran microcrustaceans (*Daphnia magna, Daphnia similis*, *Hyalella azteca* and *Hyalella meinerti*) and fish (*Danio rerio*, *Lepomis macrochirus*, *Oncorhynchus mykiss*, *Pimephales promelas* and *Poecilia reticulata*) .Thus, the search for a species for use in toxicity tests should take into account the relatively constant sensitivity of the organism to a variety of chemical agents; the easy of cultivation and maintenance conditions; organism availability and species containing genetic stability (Hoffman *et al.*, 1995; Berthet, 2015).

1.3.1 Daphnia similis

Daphnia similis, Richard, 1894 from the family Daphniidae, order Cladocera (Figure 1. 2), is a freshwater microcrustacean widely used in freshwater ecotoxicology studies (Rodgher *et al.*, 2008; Berthet, 2015). Popularly known as a water flea, its physical features are previously rounded head, a large dark spot on the anterior extremity, compound eye and size not exceeding one millimeter in length (0.8 to 0.9 mm) (Elmoor-Loureiro, 1997). Males and females can be differentiated by their shape and size. The movement of this organism is performed by a set of two antennas and is due to this locomotion mechanism that allows the visualization of a generally vertical and irregular movement of the organism. The continuous pulsating movement creates streams of water and through the numerous bristles found on the paws the food composed of phytoplankton, bacteria and organic debris is filtered into the mouth.

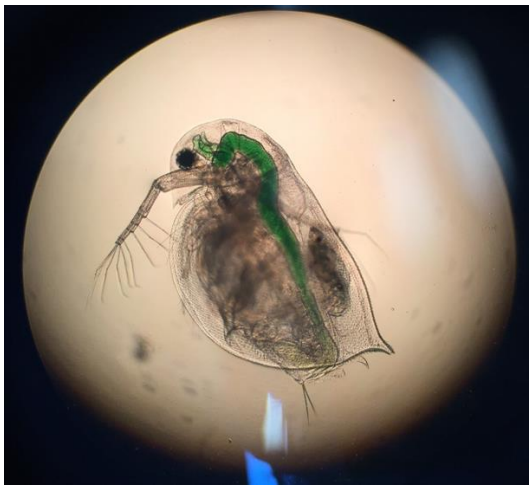


Figure 1. 2 – Water flea, Daphnia similis.

In general way, females reproduce by parthenogenesis, but in situations where conditions are not ideal for growth, males and females are produced, and sexual reproduction occurs. Fertilized eggs hatch in the form of resistant ephipium that can remain inactive until conditions are once again favorable for growth and reproduction (Dodds, 2002). Males are smaller than females, with an almost triangular shape. Freshwater invertebrates genus *Daphnia sp.* are recommended by the EPA (Environmental Protection Agency) for conducting toxicity tests in aquatic environments because of their short life cycle, genetic stability and well-established laboratory cultivation environments due to their short life cycle, genetic stability and well-established laboratory cultivation (Zagatto and Bertoletti, 2006).

1.3.2 *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, Krisitiansen & Skulberg, 1987

Raphidocelis subcapitata, formerly known as *Selenastrum capricornutum* (Abnt, 2018), is an unpolluted green pigmented unicellular alga widely used in toxicity studies because it is considered to be easy to grow and to grow rapidly (Reynolds, 1984; Hoffman *et al.*, 1995). The use of algae as a biological indicator is important because they are primary producers, where any change related to the dynamics of their communities can affect the higher trophic levels of the ecosystem (Costa *et al.*, 2008). As far as phytotoxic tests are concerned, freshwater microalgae are more commonly used than any other type of freshwater plant, but used less frequently compared to

animal species. The verification of algal growth can be determined by counting the number of cells with the aid of optical microscope, electronic particle counter, spectrophotometry or fluorimetry, or by turbidity measured at 750 nm (Abnt, 2018). The most common parameter used to evaluate toxicity using algae is related to algal growth inhibition (Epa, 2002; Standardization, 2012; Abnt, 2018). In addition, technical experience is required to conduct this type to assay and it is a restriction factor for its use (Hoffman *et al.*, 1995). Providing fundamental quantitative information related to the availability of chemicals, nutrients or potentially toxic substances and their effects on ecosystems is considered a key factor.

1.3.3 Nanoparticles ecotoxicity studies

The interactions between nanomaterials biotic components on the aquatic environment are complex and much research is necessary to understand in detail how the physical, chemical and other properties of nanomaterials influence on these interactions, and thus determine the ultimate impact of nanomaterials on the environment. Thus, environmental studies are considered a challenge for ecotoxicology due to the high complexity of a real ecosystem that cannot provide a control abiotic parameters. On the another hand, synthetic or standardized system allows more environment controlled conditions, but do not account for all real transformations.

Research during the last twenty years has confirmed that nanoscale materials manifest unexpected toxicity but, unfortunately, quite incompletely understood (Adams and Barbante, 2013). Various toxicity studies were performed with AgNPs, i.e., Asharani an co-authors (2008) have suggested that phenotypic defects in *Dani rerio* (zebrafish) are not due to Ag^+ but from AgNPs (when coated with starch and bovine serum albumin), whereas Foldbjerg et al. (2009) and Miura and Shinohara (2009) reported observations of similar biological responses due to the presence of both Ag^+ and NPs capped with polyvinylpyrrolidone (PVP). Kim et al. (2007) have determined the antimicrobial activity exposing *Escherichia coli*, concluding similar effect of those NPs comparing to silver nitrate solution. Nevertheless, as showed in the studies

previously cited or despite the use of Ag NPs untransformed, conclusions are often contradictory.

Very important is also consider the NPs introduction in the food chain within the aquatic system (Ferry *et al.*, 2009). This scenario requires an evaluation of ecological risk involving biomagnification due toxicological effects (Zhu *et al.*, 2010). Trophic transfer studies using TiO_2 NPs incorporated the bacteria *Pseudomonas aeruginosa* to the protozoan *Tetrahymena thermophila*, possible effects of growth of the organism were observed. Mielke et al. 2013 have compared the effects of direct absorption of TiO_2 NP on the protozoan *T. thermophila* with the trophic transfer using *P. aeruginosa*, concluding that the population and the growth of the protozoa diminished when in the presence of the bacteria already cultivated in the presence of the particles (Mielke *et al.*, 2013). Zhu et al. (2010)evidenced the direct relationship of the transfer of TiO_2 NPs using Daphnia (microcrustacean) and zebrafish (fish) in a simplified freshwater food chain. Bouldin et al. (2008) have studied transfer chains considering the microalgae (*Raphidocelis subcapitata*) and zooplankton (*Daphnia similis*), concluding that ingestion should be considered source of NPs for higher trophic levels of aquatic organisms. However, and to the best of our knowledge, there are few studies of food chains that relate the presence of AgNPs transformed with the possible toxicity and bioaccumulation generated by food transfer.

The environmental, biological and toxic issues of NPs have been considered obstacles to the development of nanotechnology. These current uncertainties need to be carefully handled to avoid public fears that block the benefits of nanotechnology. Adequate toxicity data, including the characterization of the transformations of NPs and bioavailable species containing NPs, and its relationship with specific cytotoxic effects are therefore extremely necessary before significant progress towards risk assessment and regulation of NPs (COM (2012) 572, http://www.eumonitor.eu/9353000/1/j9vvik7m1c3gyxp/vj3frr4seyy6#p3).

CHAPTER 2: CHARACTERIZATION OF DISSOLVED ORGANIC MATTER FROM RIVER WATER AND EXOPOLYSACCARIDES

Abstract

Considered a complex substance, dissolved organic matter (DOM) is ubiquitous in marine and freshwater environments. DOM has many functions that modify physical, chemical and biological processes in ecological systems. For this reason, study the characteristics of those material is essential to better understand the process in aquatic environments. The present work aims to characterize two different types of dissolved organic matter such as aquatic humic substances from river and exopolysaccarides from algae in order to better understand them behavior present in the aquatic systems. In this article aquatic humic substances, considered a refractory material portion of DOM, were extracted by chromatography process. Exopolimeric substances, another class of organic material, was obtained from *Raphidocelis subcapitata* cultures, a microalga commonly used in toxicity bioassays. Both organic matter were characterized in terms of elemental composition, titration and FTIR analysis. Based on the results obtained in the present work it is possible to conclude there are some differences between the two ubiquitous organic matter in their composition, both natural organic matter. However, even the different composition both keep structure related to binding capacity and complexation potential. Thus, the characteristic from humic material as well from EPS are in accordance with literature, presenting functional groups that will play important role in the aquatic environment.

Keywords: dissolved organic matter; exopolymeric substances; aquatic humic substances.

2.1 Introduction

Organic matter in soils, peat and sediment consists of a mixture of substances in various stages of decomposition. Formed from the biological and enzymatic decomposition of plant and animal residues generally present in soil, humic

substances (HS) represent the main class of constituents of natural organic matter (NOM), globally distributed in terrestrial and aquatic environments (Stevenson, 1994).

In general, DOM acts as an important energy source for heterotrophic aquatic organisms and as a nutrient source for autotrophs and heterotroph organisms. However, the chemical structures and sizes of the DOM components may affect DOM bioavailability and nutritive value. It is estimated that half of the dissolved organic carbon (DOC) disposed in surface and ocean waters is HS refractory organic matter type (Rocha and Rosa, 2003). According to their solubility the humic substances can be separated into three main components: humic acids (non-water soluble portion at pH below 2), fulvic acids (soluble portion at any pH condition) and humine (water-insoluble portion independent of pH) (Maccarthy, 1990).

Furthermore, HS have characteristics such as: binding to numerous organic substances, formation of complexes with metallic ions and colloids, influences the brown or bronze coloration of water when in high concentrations (acting on light absorption and influencing the *processes* of photosynthesis) and resistance to biological degradation. In the aquatic environment, metals can complex with dissolved organic matter, especially humic substances. These represent 80 % of organic matter dissolved in natural waters and influence numerous biogeochemical processes (Costa *et al.*, 2008). The interaction between the functional groups of humic substances and metals occurs according to the affinity of the bonds formed between these species. This affinity can range from weak forces of attraction to the formation of highly stable coordinate bonds. Studies of processes capable of reducing the concentration of free metal ions such as complexation reactions show the possibility of significant decrease in toxicity. Bezerra et al. (2009)when performing tests with natural organic matter (humic and fulvic acids) highlights the competition for binder sites in natural environments for metal ions. Thus, direct correlation of organic matter *versus* ligand concentration needs to be investigated in detail.

Extracellular polymeric substances (EPS) matrix is formed by different biochemicals secreted by microbes, release of cellular material/products by cell lysis or organic matter in the medium resulted from physiological activities of microorganisms in natural environments. Exudates from algae, mainly consisting of organic acids of low molecular weight and extracellular polysaccharide, play a significant role in geochemistry processes including reduction of metals, the rate and

extent of dissolution of minerals and can thus also affect the fate of NPs in the environment (Seders and Fein, 2011). In this context, the present work aims to characterize two different types of dissolved organic matter such as aquatic humic substances from river and exopolysaccarides from algae in order to better understand them behavior present in the aquatic systems.

2.2 Material and Methods

2.2.1 Aquatic Humic Substances: study area, sampling and extraction

2.2.1.1 Water sampling and characterization

Humic substances were sampled from Sorocabinha River (Iguape/SP -Brazil, S 24°41′59″S, W 47°33′05″), hydrographic region known for the presence of high mangrove areas and high amount of NOM as observed in Figure 2. 1.

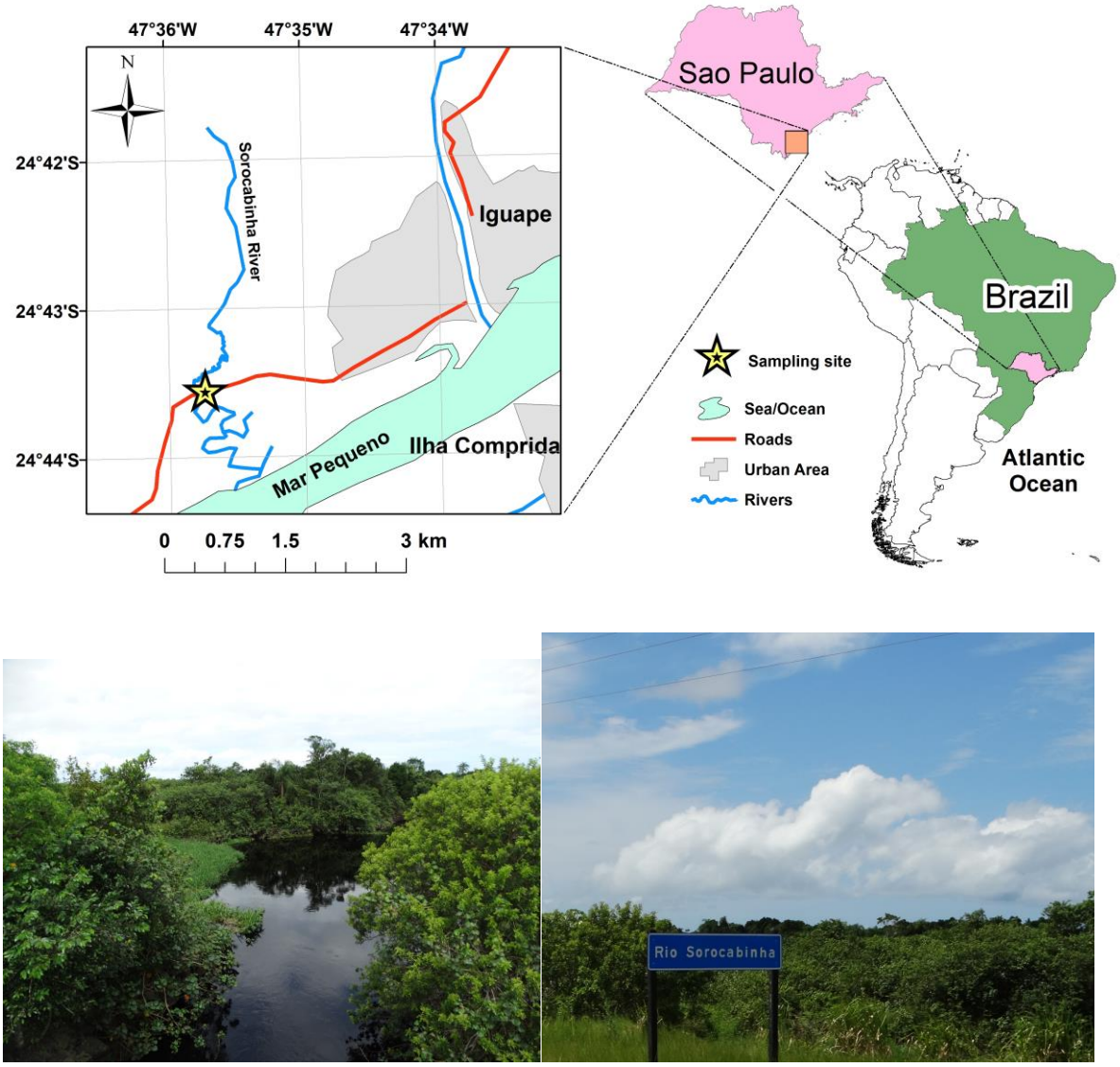


Figure 2. 1 - Localization map (up) and ilustrative pictures (down) of the sample point area (Iguape-SP) for Aquatic humic substances. Sampling date: March 15, 2015.

Physicochemical parameters measurements such as electrical conductivity, pH and dissolved oxygen (DO), in situ measurements and, in laboratory, biochemical oxygen demand (5 days of incubation at 25 °C (BOD_5) were performed. The amount of metal presented in the *in natura* water were quantified following the procedures from recommended literature (Usepa, 1992). Thus, to quantify the total metal (TM) concentration, 50 mL of water sample were digested. In the same way, water sampled were filtered using a vacuum pump with 0.45 µm filter (Sartorius) for dissolved metals (DM) concentration, both in triplicate. Sub-boil distilled nitric acid (Anidrol P.A.) was added to each sample and then heated to 95-100 °C in a heater plate, until reduction

of the digested contents (around 10 mL). Each sample was adjusted with ultrapure water in 25 mL volumetric flask. In order to verify the free metal plus small complexes < 1kDa portion an ultrafiltration (UF) system with ultrafiltration cells and cellulose regenerated membrane (Millipore®, pore size of 1 kDa and diameter of 76 mm) was used. The ultrafiltration system was coupled to a peristaltic pump (Gilson) by Tygon® tubes. Initially, a cleansing procedure was performed using approximately 50 mL of ultrapure water that was permeated throughout the system. Then, 50 mL of HCl solution 0.001 mol L^{-1} followed by more 50 mL of ultrapure water, 50 mL of 0.1 mol L^{-1} NaOH solution and again 50 mL of ultrapure water. After all the steps, the maximum residue from the cleansing was withdrawn before start the sample ultrafiltration. For this purpose, 100 mL of sample was ultra-filtered by removing an aliquot of 2 mL of post-filtered solution, stored in Eppendorf and acidified with HNO_3 solution (20 %). The amount of Ag, Al, As, Cd, Pb, Co, Cu, Sr, Fe, Mg, Mn, Ni, P, Sr, K and Zn were quantified by ICP-OES (Agilent, 720 series).

2.2.1.2 AHS extraction

The schematic procedure of HS extraction is presented in Figure 2. 2. The HS were extracted following recommendated literature (International Humuic Substances Society, IHSS; Thurman and Malcolm, 1981) using chromatographic method with column packed resin Superlite™ DAX-8 (Sigma Aldrich), the most common procedure used for AHS isolation from aqueous samples (Abbt-Braun et al., 2004). Briefly, The sampled water were acidified at low pH values (pH < 2) using HCl M and the humic substances were retained in the resin when the water passed through the XAD-8 column. The AHS was desorbed using Sodium Hydroxide (P.A. Dinamica) at 0.1 mol L^{-1}. The humic substances extracts were acidified to pH value of 5.8 (according to in situ measurement) using HCl (P.A. Synth) 0.1 mol L^{-1}. and stored for posterior use.

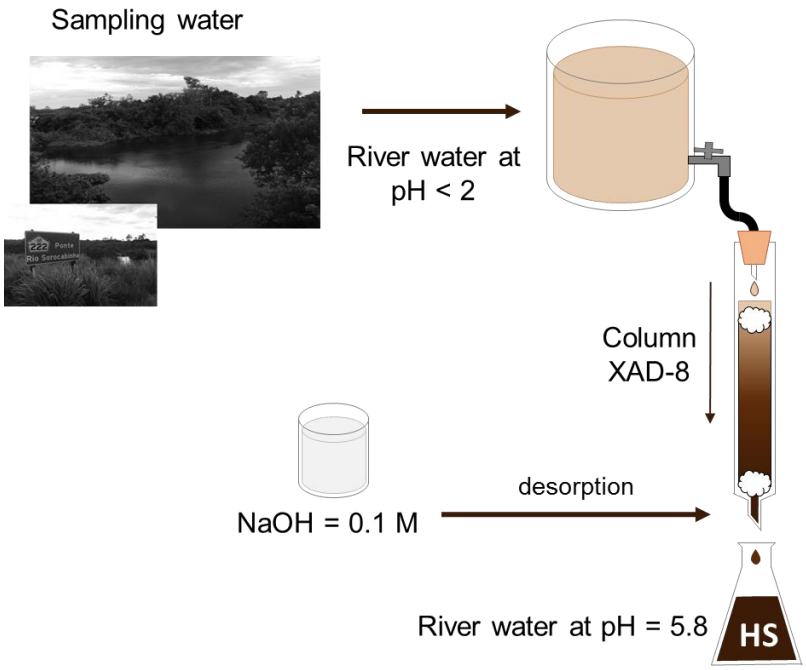


Figure 2. 2 - General schedule for HS extraction.

Before every use, a resin cleansing process was performed: first the resin was left in metanol (P.A. Synth) in agitation for 2 hours. The system resin + metanol was washed extensively with ultrapure water until obtain pH 7. Sequentially, HCl 0.1 mol L^{-1} was added and left in agitation for 24 h and again washed with ultrapure water. Finally, the same was done but adding NaOH mol L^{-1}.

2.2.2 Exopolysaccharides collection (extraction)

2.2.2.1 Microalgae culture

Raphidocelis subcapitata were growth in L.C. Oligo Medium following national recommended literature (Abnt, 2018). LC Oligo medium was prepared using specific solutions containing nutrients selected for growth development of algae was chosen. The composition of each solution as well the volume used for preparing 1 L of culture medium are set out in .

Laboratory adaptations were performed in order to enhance the microalgae cultivation condition, avoid contamination of the strains acquired as well to standardize the EPS production. Thus, the microalgae were cultivated in incubator with

temperature and controlled photoluminescence. During the algae growth, laminar flow cabinet with UV lamp was used and the materials were sterilized using the autoclave. The determination of algae concentration and monitoring the contamination control of the cultures were obtained by using optical microscope and also by espectrophotometry, the last one more described in Table 2. 1.

Table 2. 1 - LC Oligo Medium composition. Source: ABNT (2018).

Solution	Reagent	Mass/mg	Preparation	Volume*/L
1	$Ca(NO_3)_2$. $4H_2O$	4.0	To dissolve and complete to 100 mL in ultrapure water	1.0
2	KNO_3	10.0	To dissolve and complete to 100 mL in ultrapure water	1.0
3	$MgSO_4.7H_2O$	3.0	To dissolve and complete to 100 mL in ultrapure water	1.0
4	K_2HPO_4	4.0	To dissolve and complete to 100 mL in ultrapure water	1.0
5	$CuSO_4.5H_2O$	0.03	To dissolve and complete to 100 mL in ultrapure water	0.5
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.06		
	$ZnSO_4.7H_2O$	0.06		
	$CoCl_2.6H_2O$	0.06		
	$Mn(NO_3)_2.4H_2O$	0.06		
	$C_6H_8O_2.H_2O$	0.06		
	H_3BO_3	0.06		
6	$C_6H_5FeO_7.5H_2O$	1.625	To dissolve and complete to 100 mL in ultrapure water	0.5
	$FeCl_3.6H_2O$	0.625		
	$FeSO_4$. $7H_2O$	0.625		
7	$NaHCO_3$	15	To dissolve and complete to 100 mL in ultrapure water	1.0

* Volume to prepare 1 liter L.C. Oligo medium.

After L.C. Oligo medium preparation, the solution was kept under stirring for 1 hour and the pH adjusted to the value around 7.0, using hydrochloric acid (0.1 mol L^{-1} and HCl 0.01 mol L^{-1}) or sodium hydroxide (0.1 M NaOH, 0.01 M) when necessary. The medium was autoclaved at temperature of 121 °C for 15 minutes. The medium was considered ready for receive the strain when it reached the environmental temperature (23 - 27 ºC).

The *R. subcapitata* strain was acquired from the Botany Department - Federal University of São Carlos (UFSCAR- Brazil). Upon receipt of the strain, splits were transferred every three days, starting initially in 100 mL Erlenmeyer containing 30 mL of culture medium, until the desired volume of work batch cultures in 2 L glass Erlenmeyer flasks was reached. Every 30 days the strain was transferred to a new medium to keep the initial strain culture during the period of the execution project.

2.2.2.2 Stablishing growth curve

In order to stablish optimal EPS production and healthy cells, experiments relating Dissolved Organic Carbon (DOC) *versus* exposition time (t_{exp}) *versus* algal concentration were performed. In triplicate, batch cultures of *R. subcapitata* were carried out in Erlenmeyer flasks containing 2 liters of autoclaved Oligo medium, in aeration system arranged in an incubator with light control (constant), temperature between 23 and 27 °C time of exposition = 20 days (Figure 2. 3).

a) b)

c) d)

Figure 2. 3 - Aspects of R. subcapitata cultures during experiment a) 1st day experiment b) 4th day experiment c) 7th day experiment d) 20th day experiment.

In order to obtain the start experimental concentration of cells (10^4 cells mL^{-1}), the initial cells concentration was collected from stock algal solution in exponential phase and the volume of stock solution was determined using the Equation 1, where V_i = stock solution volume (mL), V_f = final solution volume (mL), C_i = initial concentration (cells mL^{-1}) and N = number of cells in suspention (cells mL^{-1}).

$$V_i = \frac{C_i * V_f}{N} \qquad (1)$$

Alternatively, a simple system using spectrophotometer was also used to calculate cells concentration. More details are disposed in the Appendix 1. The organic carbon and number of cells were monitored during t_{exp} = 0, 1, 2, 3, 4, 5, 6 ,7, 10, 11,12 and 20 days. Dissolved Organic Carbon was determined as following: 15 mL of algal solution was taken and centrifuged to 3,500 rpm for 15 minutes. The supernatant was taken into previously decontaminated flasks and stored (4-10 ºC). The DOC was determined by high oxidation temperature (850 °C) using a Carbon Analyzer (multi N / C 3100, AnalytikJena). Potassium biphthalate (PA., Synth) was used as organic carbon standard and carbonate and sodium bicarbonate were used as standards for inorganic carbon.

Figure 2. 4 shows the results obtained from the organic carbon production experiment and number of cells over time. After 12 days there is still an increase in the number of cells and organic carbon with an increase of 2 mg L^{-1} between 15th and 20th day of culture. Studies involving exudates production carried out by Guéguen et al.

(2003) proposed collect the algae excreted produced after 7 days of experiments. However, the data obtained in the experiment show that the maximum EPS production can occur over a longer period of exposure. In this way, a period of 15 days of cultivation was pointed as the exposure period for the EPS production, to follow the procedures to obtain the EPS to perform the tests.

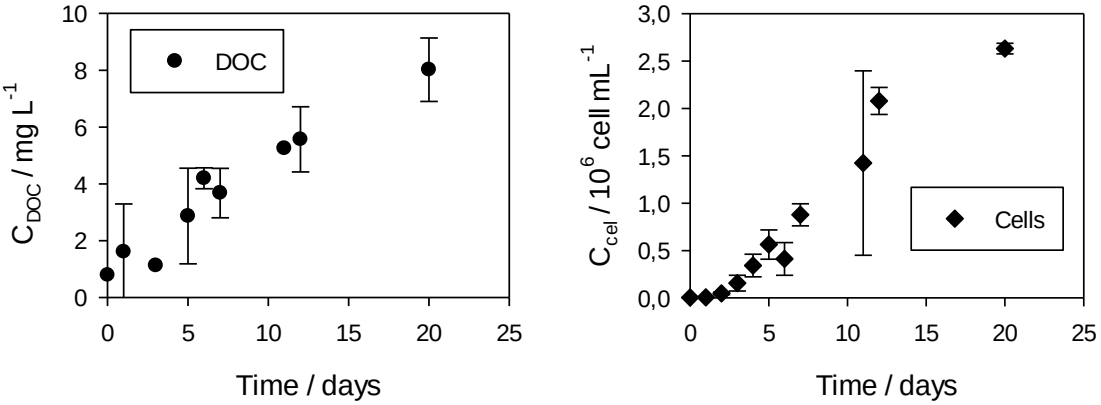


Figure 2. 4 - Growth curve of R. subcapitata in terms of cells and organic carbon versus time.

2.2.3 EPS stock preparation

In order to obtain the EPS, *R. subcapitata* batch cultures were obtained as already described in the previous section. The general procedure is outlined in Figure 2. 5. After 15 days, the algae solution was centrifuged for 15 minutes at 3,500 rpm, the supernatant was carefully removed and the cells discarded properly. In sequence, then the solution was filtered using 0.45 µm filter (Sartorius) for removal of possible remaining cells and organic material > 0.45 µm. The filtrate was subjected to a dialysis procedure to remove metals and the EPS solution was freeze-dried when necessary or kept in solution for situations such as the EPS and NPs interaction tests. For the latter case, in order to determine DOC concentration, the carbon concentration was determined by carbon analyser.

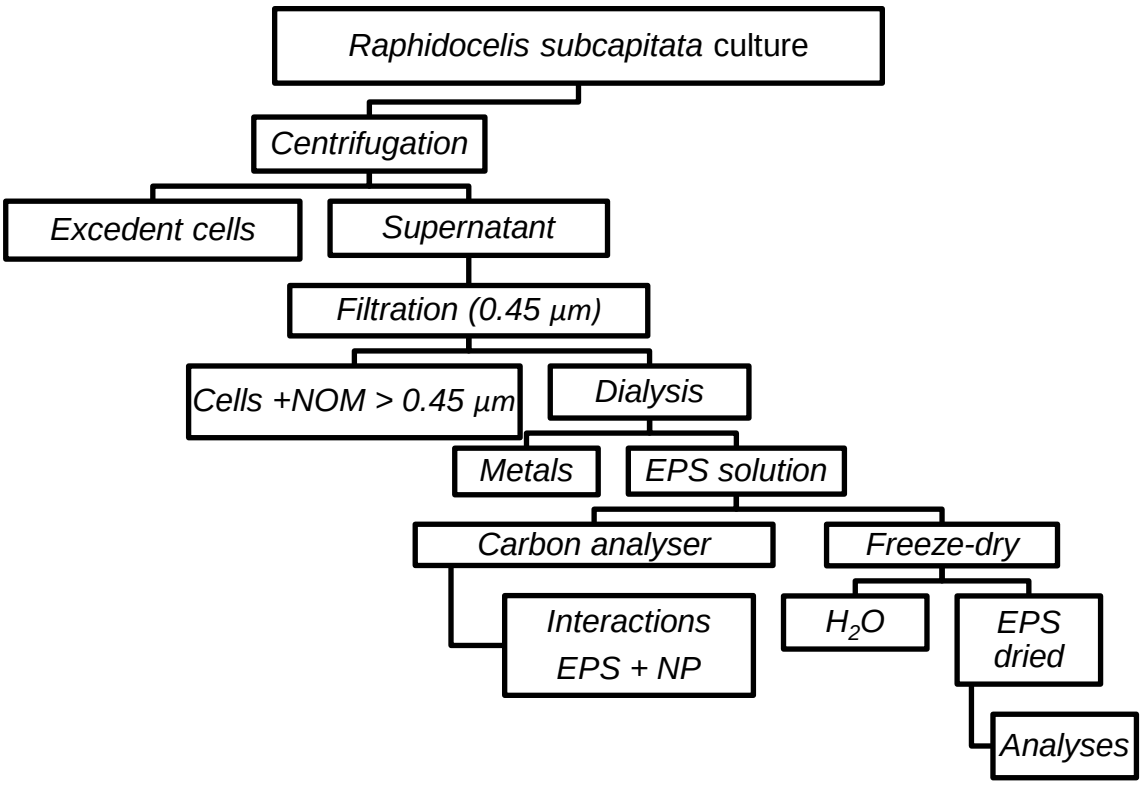


Figure 2. 5 - General steps for EPS production.

2.2.4 NOM characterization

2.2.4.1 Presence of metals

The concentration of metals (aluminum, cadmium, copper, cobalt, chromium, strontium, iron, magnesium, manganese, nickel, silver and zinc) from HS and EPS is controlled by a sample digestion using USEPA (Usepa, 1996). Dried organic matter (HS and EPS) were digested in triplicates, (around 0.1 g of material) adding hydrochloric and nitric concentrates and then concentrated hydrogen peroxide, leaving the samples in heating plate not exceeding the temperature of 95 ºC. After digestion, the volume was adjusted and the metal concentration were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES).

2.2.4.2 Elemental analysis

The carbon and nitrogen contents into the organic matter were measured using Organic Elemental Analyser (Thermo Scientific, Flash 2000). Freeze-dried organic matter samples (3-10 mg) were weighed in universal soft tin containers (Thermo Scientific, vol. 157 µL) using microbalance (Mettler Toledo, MX5). The samples were subjected to combustion procedure and the calibration curve was performed using BBOT (2,5 Bis (5-tert-butyl-benzoxazol-2-yl) thiophene $C_{26}H_{26}N_2O_2S$) (Thermo Scientific) was used as standard for calibration curve determination.

2.2.4.3 Potentiometric titration

In order to characterize the type and concentration of functional groups present in the HS and EPS acid-base titrations were performed. For this purpose, 15 mL of Milli-Q water plus 15 mL of $NaClO_4$ 0.01 M solution were transferred in a reactor in order proceed with a calibration only with initial conditions. The titration system (as shown in the Figure 2. 6) is composed by a reference electrode, using nitrogen (N_2) atmosphere. After obtain the calibration titration, the same amount of 0.01 M $NaClO_4$ and Milli-Q water together with HS or EPS in a concentration of 1.0 g L^{-1} in terms of mass was titrated in the 5 different ionic strengths (0.005, 0.01, 0.1, 0.3, 0.5 M). The electrostatic parameter model were obtained using acid-base potentiometric titration data, using at least 3 Ionic strengths (I.S.). FIT (Kinniburgh, 1993) was used for NICA-Donnan parameters adjusts modelisation.

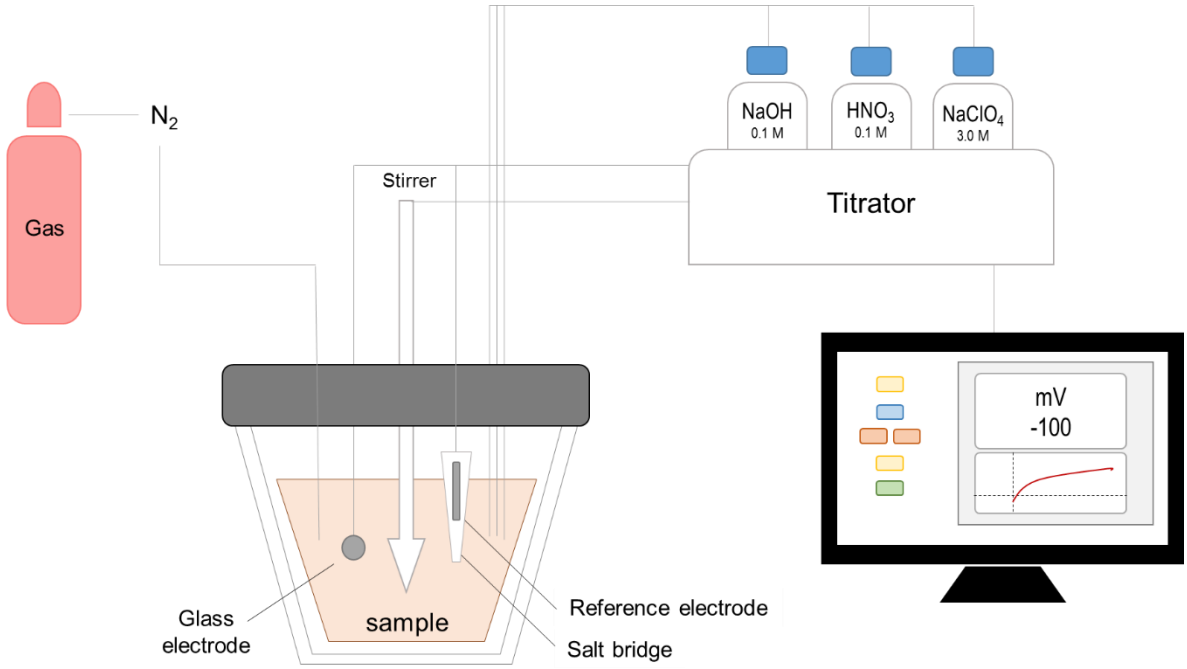


Figure 2. 6 - General schematic titration system used for phenolic and carboxylic groups determination.

2.2.4.4 Fourier Transform infrared spectra (FT-IR)

Fourier Transformed Infrared (FT-IR) is a qualitative technique applied to characterize organic matter in terms of functional groups (Abbt-Braun *et al.*, 2004). For this purpose, both natural organic matter solutions were freeze-dried and the samples in powder were analyzed by FT-IR (Perkin Elmer, Spectrum 65), using the follow conditions: resolution (4 cm^{-1}) and spectral range (4000 and 650 cm^{-1}).

2.3 Results and Discussion

2.3.1 Sorocabinha river water characterization

The values referring to the *in-situ* measurements of Rio Sorocabinha are disposed inTable 2. 1.

Table 2. 2 - Characterization of surface waters of Sorocabinha River, Sao Paulo, Brazil.

Parameter	Meausurement

Electrical conductivity (µS cm^{-1})	136.0
DBO_5 (mg L^{-1})	0.54
OD (mg L^{-1})	1.91
pH	5.8
Temperature (ºC)	23.5
TOC (mg L^{-1})	56.9

The values Sorocabinha shows a receptor body with impact potential, justified by the presence of high amounts of organic matter. Accordin to Rocha and Rosa (2003) can be observed through the characteristic dark coloration. The level of dissolved oxygen in the water is below the limits established by CONAMA (2005) which indicates concentrations above 5 mg L^{-1} as satisfactory for dissolved oxygen.

It was possible to obtain the distribution of the total, dissolved and free metal values as observed in Figure 2. 7. The chemical elements Al, Ca, Fe, K, Mg and Mn at high concentrations and at concentrations of a few micrograms per liter, Cr, Cu, Ni, Sr and Zn. Most of the detected metals distributed in the dissolved fraction and a small portion in their free form or in small complexes are observed.

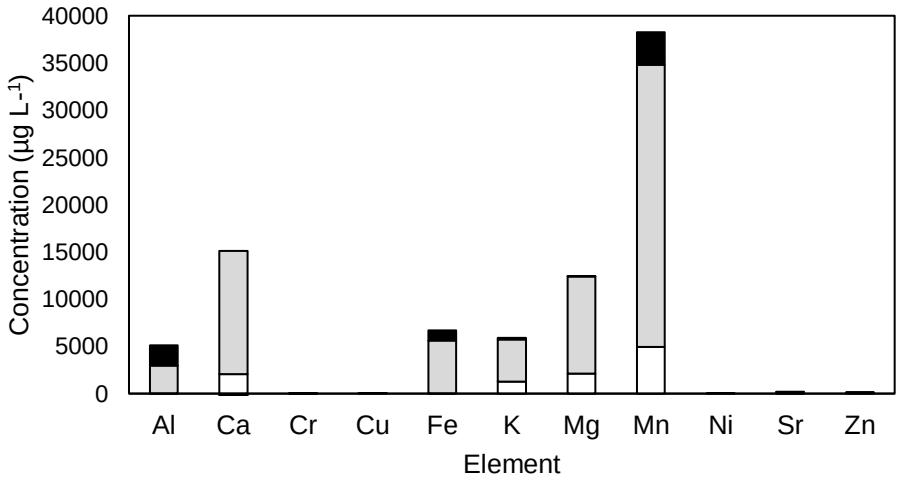


Figure 2. 7 - Results of measurements of metals present in the water collected to obtain AHS extracts. Black bar = complexed metals; gray bar = dissolved metal; transparent bar = free metal plus small complexes lower than 1 kDa.

The fact that the metals are mostly distributed in the dissolved part up to 1 kDa, shows strong evidence of those elements associated with the humic material. Rosa et

al. (2007) have found metals such as manganese, copper, cadmium and nickel observed in waters rich in humic hydrocolloids in them study.

The relevance of the study of the distribution of the metals in free and dissolved fractions complement studies of bioaccumulation of trace elements. Meylan et al. 2004 have showed that the element bioaccumulation varies according to the concentration of free ions. In addition, Bradac et al. (2009) states that binders present in the environment, such as HS present in the aquatic environment in the dissolved fraction, may contribute to the movement of metals in microalgae, indirectly affecting other aquatic organisms, for example due to trophic transfer chains. Thus, the results of metals in water samples, which have the NOM to be studied, contribute to establish hypothesis related to the interaction of HS and interactions to be carried out together with metallic nanoparticles.

2.3.2 NOM characterization

The results of digestion AHS and EPS are given in the Table 2. 3. The humic material has high concentrations of Al and Fe. The presence of metals such as manganese, copper, cadmium and nickel are commonly observed in waters rich in humic hydrocolloids (Rosa *et al.*, 2007). Silver was always below detection limit (i.e. give here number) in both HS and EPS extract.

Table 2. 3 - Metal composition of AHS and EPS

Element	Concentration (mg kg^{-1})	
	EPS	HS
Ag	<LOQ	<LOQ
Al	3.8±0.6	177.3
As	<LOQ	<LOQ
Cd	<LOQ	<LOQ
Co	<LOQ	<LOQ
Cr	<LOQ	0.251±0.001
Cu	<LOQ	<LOQ
Fe	<LOQ	1051.76±14.7
Mn	<LOQ	7.1±0.4
Ni	2.95±0.003	<LOQ
Pb	<LOQ	<LOQ

LOQ = Limit of quantification

The C and N contents in the samples showed 43.95±0.02 % and 0.53±0.01 % for AHS and 34.0±0.1 % and 20.5±0.1% for EPS, respectively. For HS, the carbon contents, we have found, are in agreement with the literature data. Normally, humic substances are characterized by a C percentage in a range of 40-60 %. However, low amounts of nitrogen were found in the AHS extracted from Sorocabinha River. In the case of EPS, Tonietto et al. (2014) have found similar contents of nitrogen in the EPS exudate composition in fractions obtained from *Mycrostis aeruginosa*.

Good master curve has been obtained using the Donnan model for the Sorocabinha AHS data as well as Benedetti et al. (1996) obtained in them data. For the EPS potentiometric titration data, as shown in Figure 2. 8, the m_{eq}/g (0.10 to 0.15) was not enough to construct a master curve because of the amount of EPS (in therms of organic carbon) is lower than necessary comparing with the calibration titration. So titration data at pH < 3.5 or pH > 10.0 were considered unreliable, and were not used for calculations. New potentiometric titrations using high EPS amounts will be performed.

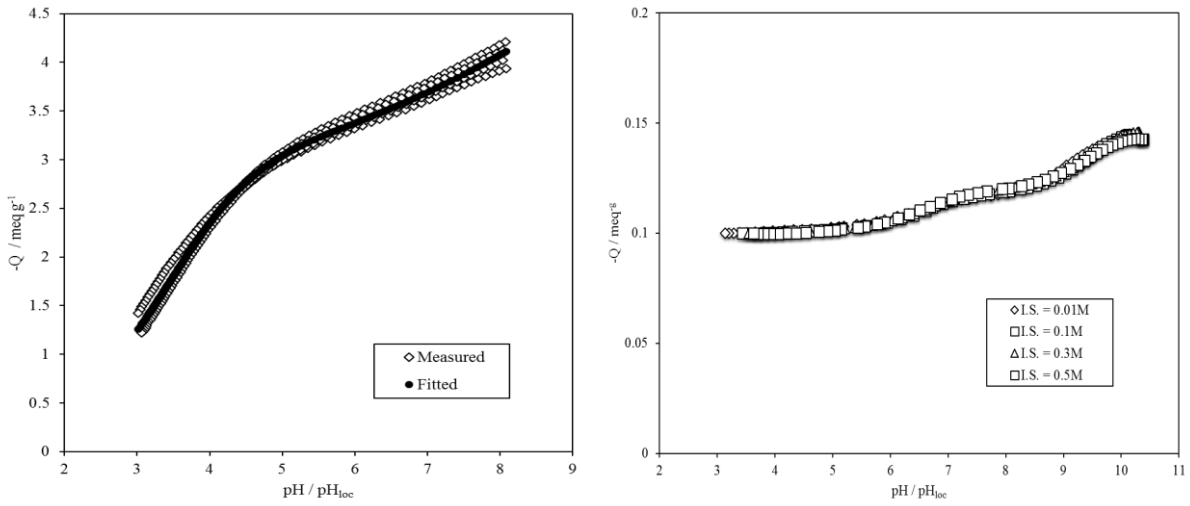


Figure 2. 8 a) Master curve obtained by the Donnan (NICA) model (closed symbols) together with the experimental charge/pH curves (open symbols) for Sorocabinha HS; b) plotted data from EPS potentiometric titrations in I.S. = 0.01, 0.1, 0.3 and 0.5 M.

Table 2. 4 shows the best fitted parameters describing the HS using NICA-Donnan model. The coefficient of determination (R^2) was 0.994. The results obtained within this study emphasizes that the well-characterized HS can be used as an analogue types of studies in natural environments(Vermeer, 1996). Donnan (NICA)

model parameters represent the carboxyl and phenolic OH group contents ($Q_{max,1}$ and $Q_{max,2}$, respectively), mean values of affinity distributions for proton binding by carboxyl groups and phenolic OH groups (log K_1 and log K_2, respectively), and the width of proton-affinity distributions of carboxyl and phenolic OH groups (m_1 and m_2, respectively). Empirical parameter describing how the Donnan volume varies with ionic strength.

Table 2. 4- Assessed parameters describing the HS using NICA-Donnan model.

Carboxylic site			Phenolic site			NICA-Donnan
log K_1	m_1	Q_{max1}	log K_2	m_2	Q_{max2}	
3.816	0.589	2.705	12.786	0.192	3.055	Data acquired

Carboxyl and phenolic OH groups contents (Q_{max}) obtained by Milne et al. (2001) have found similar parameters. The functional groups that may be present in fulvic acid are carboxyl, phenolic hydroxyl, alcoholic hydroxyl and carbonyl. The functional groups vary with the particular acid sample. Approximate ranges in units of milliequivalent per grams of acid are: total acidity: 12 to 14; carboxyl, 8 to 9; phenolic hydroxyl, 3 to 6; alcoholic hydroxyl, 3 to 5 and carbonyl, 1 to 3. In addition, some methoxyl groups, $-CH_3$, may be encountered at low levels (Manahan, 2005).

The FTIR peaks were assigned based on the existing literature. According to transmittance spectrum acquired (Figure 2. 9). AHS present broad peak near to 3400 cm^{-1} related to O-H stretching (H-bonds) of alcohols, phenols and carbon acids and/or -NH proteins (Burba *et al.*, 1995; Abbt-Braun *et al.*, 2004; Sun *et al.*, 2009; Zeng *et al.*, 2016). The domain 1700-1400 cm^{-1} is attributed to -COOH groups and the peak 1570 cm^{-1} could be related to asymmetric stretching of COO- (Terkhi *et al.*, 2008) and/or N-H bending in amino acids of proteins (Zhou *et al.*, 2016). Infrared peak at 1380 cm^{-1} was assigned to -OH, C-O stretching (phenols, alcohols, carboxylic acids) and CH(aliphatic) and symmetric stretching of COO- (Burba *et al.*, 1995; Rocha and Rosa, 2003; Terkhi *et al.*, 2008). The peaks between 950-1170 cm^{-1} might be represented by the presence of polysaccharides. Abbt-Braun et al. (2004) have justified the presence of polysaccharides in the humic substances because of incomplete removal during the isolation procedure leading to the occurrence of carbohydrate structures in the composition of humic substances.

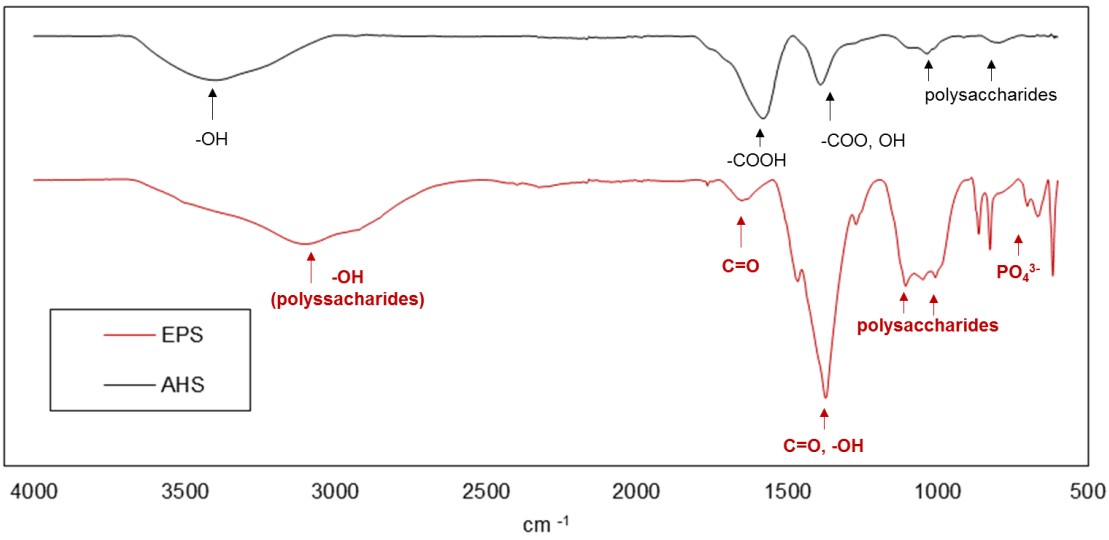


Figure 2. 9 - Fourier transform infrared spectra of AHS extracted from Sorocabinha River (up black) and EPS extracted from algae Raphidocelis subcapitata (down red).

For EPS spectra, the broad peaks around 3400 and 2900 cm^{-1} can be attributed to OH- stretching vibrations of polysaccharide (H-bonds) and aliphatic -CH stretching (CH_3-, CH_2-, CH) (Abbt-Braun *et al.*, 1989; Sun *et al.*, 2009; Zeng *et al.*, 2016). Similar peak at 2920 cm^{-1} were reported for (Sun *et al.*, 2009) characteristic of alkyl chains presented in EPS spectra. The peak at 1630 cm^{-1} indicates C=O stretching vibration (ketones, amides, quinones) from proteins (Abbt-Braun *et al.*, 1989; Sun *et al.*, 2009; Zeng *et al.*, 2016; Zhou *et al.*, 2016; Zheng *et al.*, 2019). The band at 1440 cm^{-1} might be associated to -CH bending vibrations (Sun *et al.*, 2009; Zeng *et al.*, 2016) and/or deformation vibration of -CH in proteins and lipids (Zheng *et al.*, 2019) contribution from an amide III band (Sun *et al.*, 2009). The strong peak around 1370 cm^{-1} might be related to C=O stretching from carboxylate (Zheng *et al.*, 2019), -OH, C-O (phenolic) and/ or CH (aliphatic) (Burba *et al.*, 1995). The peak at 1268 cm^{-1} indicates bending of N-H group and the stretching of the C-N groups in Amide III present in proteins (Zheng *et al.*, 2019). Spectra band between 1150 and 1000 cm^{-1} could be attributed to the presence of polysaccharides (Burba *et al.*, 1995; Sun *et al.*, 2009; Zeng *et al.*, 2016). Sun et al . (2009)have found in the same spectra range associated to polysaccharide groups for the EPS obtained. The peaks between 600 and 900. Alson, they have found peaks in the region around 400 and 900 cm^{-1} might to be assigned to phosphate which composes the nucleic acids. (Zeng *et al.*, 2016) have attributed the same zone to the presence of phosphate or sulfate groups.

The chemical moieties of the HS and EPS provided by spectroscopic analyses is in agreement with literature data since humic substances are normally associated to the presence of specific groups such as alcohols, phenolic groups, carboxylic acid groups, N-H amino acids proteins and polysaccharides. The functional groups identified through humic substances were also evidenced in previous work (Abbt-Braun *et al.*, 1989; Burba *et al.*, 1995). For EPS composition, it's possible to suggest the presence of phenolic groups, polysaccharides, proteins, lipids and sulfate/phosphate groups provided by nucleic acids. Liu and Fang (2002) have shown that EPS are composed of a variety of organic substances, where carbohydrates and proteins correspond to major components. This evidence is also reported by Tonietto et al. (Tonietto, 2014) that have analyzed exudates from the algae *Cylindropermopsis raciborskii*, attesting the presence of carbohydrate, lipids and organic and amino acids as constituents of the organic material. Zeng et al. (2016) have reported in their work that proteins and polysaccharides accounted for significant proportions in EPS and, in addition, each content depends of the processing involving the organic matter production.

2.4 Conclusions

Based on the results obtained in the present work it is possible to conclude there are some differences between the two ubiquitous organic matter in their composition, both natural organic matter. The results have showed AHS might be associated groups: phenolic groups, carboxylic acid groups, alcohols. EPS associated groups were phenolic groups, polysaccharides, proteins, lipids and sulfate/phosphate groups. Carbohydrates and proteins correspond to major components of excretades. However, even the different composition both keep structure related to binding capacity and complexation potential. Thus, the characteristic from humic material as well from EPS obtained are in agreement with literature and the presence of functional groups with binding bility can play important role in the aquatic environment dynamic.

CHAPTER 3: FATE OF SILVER CITRATE (CIT) AND POLYETHYLENE GLYCOL (PEG) CAPPED NANOPARTICLES IN AQUATIC SYSTEM: INTERACTIONS WITH NATURAL ORGANIC MATTER

Abstract

Despite the growing interest and use of nanoparticles (NPs), the gathered knowledge on their environmental consequences is still scarce, since the large majority of the studies do not consider the effect of the presence of natural coatings around the particles. In order to better understand the fate of NPs in aquatic systems, in this work the approach was to determine the fate of silver NPs capped with citrate and polyethylene glycol dispersed in ecotoxicological matrices in the presence of environment relevant natural components such as humic substances (HS) and extracellular polymeric substances (EPS). The interactions between NOM-AgNPs were assessed by ultracentrifugation to obtain the Ag dissolution rate and Electrophoretic mobility (EPM). For dissolution response, it is possible to observe the influence of both EPS and AHS in the decrease of dissolution rate. Furthermore, the presence different types of organic matter (EPS and AHS) provokes a NP stabilization in the medium. The amounts of DOC and particle concentration affect the dissolution rates that also depends of the coating type (citrate or PEG). Exposure medium (AM and DM) also affect in the AgNP transformations showing the importance of studies taking to account environmental relevant matrices. Based on the investigation performed in the present work it's reasonable to verify the influence of the fate of silver nanomaterials in the bioavailability and consequently helping in the toxicity prediction. This approach will allow a breakthrough in the understanding of environmental and biological fate of nanomaterials and will certainly have a considerable impact on regulatory guidelines.

Keywords: water; nanomaterial; environmental chemistry; silver nanoparticles; exopolysaccarides; humic substances.

3.1. Introduction

The current expansion of nanoscience and nanotechnology and the massive use of NPs manufactured particles, that have at least one dimension in the nanoscale (1-100 nm), in commercial products could be mirrored by an increase of these materials in the biosphere. NPs are not only small crystals but an intermediate state between molecular and bulk materials exhibiting unique magnetic, electrical, optical,

mechanical, chemical and structural properties resulting from the high ratio between surface area and volume, corresponding to a high percentage of surface atoms. These unique properties result in the application of NPs in a wide variety of areas, including medical, environmental, computer, textile and energy (Ju-Nam and Lead, 2008).

Also, one important aspect controlling their environmental fate is related to the presence of coatings around the nanoparticles, whether they are manufactured and/or natural. The first one is the result of the addition of stabilizers by the manufacturers in order to improve the mobility of the particles and stabilization in terms of size and the latter resulting in interactions between environmental and biological constituents. Thus, the highly dynamic behavior of NPs in environmental systems results in a set of physical and chemical transformations. A large number of NPs may be composed or contain components able to undergo (i) reduction and/or oxidation (Derfus *et al.*, 2004; Lok *et al.*, 2007; Shi *et al.*, 2011), (ii) photoreaction (Zhang *et al.*, 2007), iii) dissolution and sulfidation (Heinlaan *et al.*, 2008; Li *et al.*, 2011; Domingos, Rafiei*, et al.*, 2013; Kaegi *et al.*, 2013) and (iv) homo (including the same NPs) and/or heteroaggregation (between NPs and another colloid) (Domingos *et al.*, 2009; Hotze *et al.*, 2010; Domingos, Rafiei*, et al.*, 2013).

The capped nanoparticles are unique emerging materials, receiving attention from the scientific community, due to their fate, behavior and toxicity still not clear. Indeed, quite significant effort from the academic and industrial community around the world converge studies in a solid framework for the assessment of nano safety and mitigate the risks associated with NPs when disposed in the environment (Klaine *et al.*, 2008). In this context, factors to be considered in the environmental risk assessment of NPs include (i) the shape of the NP that will be present in commercial products, including the presence of manufactured coatings, (ii) the potential that the material tends to be released in the environment, and (iii) the transformation of the material that may affect exposure. This complexity is not unlike the problems associated with exposure of metal dissolved in water since depending on the chemistry of the aquatic ecosystem the metals can have different oxidation states, speciation and therefore different degrees of bioavailability.

One of the most critical transformations of NPs and in a way by now neglected is the adsorption of natural colloids, including natural organic matter (NOM), such as exopolysaccarides (EPS) and aquatic humic substances (AHS). These natural entities

can bind to the surface of the NPs forming a coating around these materials, affecting its physical-chemical and biological identity, and thus, affecting its reactivity surface and interactions with cells and organisms. The effects produced by NOM are complex and difficult to predict, however, it is extremely important to exploit these interactions as the NOM concentrations are orders of magnitude higher than those NPs concentrations and therefore quite likely to modify the properties substantially behavior of NPs (Labille and Brant, 2010). Then, the approach of the present work was evaluate the fate of silver NPs capped with citrate and polyethylene glycol dispersed in ecotoxicological matrices in the presence of environment relevant natural components such as aquatic humic substances (AHS) and extracellular polymeric substances (EPS) by electrophoretic mobility, dissolution and speciation experiments. or understanding solubilization and charge effects of NPs transformed into a well-planned array of environmentally representative freshwaters.

3.2. Materials and Methods

Commercially available chemical and solvents of Analytical purity were used to perform the experiments. Ultra High Purity water with maximum resistivity of 18.2 M Ω cm^{-1} was used throughout the experiments. Test medium typically used for ecotoxicology studies, as Oligo Media and soft reconstituted water were prepared based on the guidelines presented in the protocols for microalgae *Raphidocelis subcapitata (Usepa, 2002; Abnt, 2018)* and microcrustacean *Daphnia sp*. (Oecd, 2004; Abnt, 2016). Algae medium (AM) and Daphnia medium (DM) element concentration are given in Table 3. 1 and Table 3. 2, respectively.

Table 3. 1 - Composition of Algae medium exposed solution.

Reagents	VStock /L	VT /L	C /M
$Ca(NO_3)_2.4H_2O$	0.001	1	1.69E-04
KNO3	0.001	1	9.89E-04
$MgSO_4.7H_2O$	0.001	1	1.22E-04
K_2HPO_4	0.001	1	2.30E-04
$CuSO_4.5H_2O$	0.0005	1	6.01E-08
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0005	1	2.43E-08

$ZnSO_4.7H_2O$	0.0005	1	1.04E-07
$CoCl_2.6H_2O$	0.0005	1	1.26E-07
$Mn(NO_3)_2.4H_2O$	0.0005	1	1.20E-07
H_3BO_3	0.0005	1	4.85E-07
$C_6H_5FeO_7.5H_2O$	0.0005	1	2.43E-06
$FeCl_3.7H_2O$	0.0005	1	1.08E-06
$FeSO_4.7H_2O$	0.0005	1	1.12E-06
$NaHCO_3$	0.001	1	1.79E-04

Table 3. 2 - Composition of Daphnia medium exposed solution.

Reagents	**VStock /L**	**VT /L**	**Cteoric/M**
Ca(SO)4.2H2O	0.022	1	1.92E-04
KCl	0.011	1	2.95E-05
MgSO4.7H2O	0.011	1	2.72E-04
NaHCO3	0.011	1	6.29E-04

3.2.1. Natural organic matter production

For EPS production, axenic culture of *Raphidocelis subcapitata* strain was acquired from Department of Botany, Federal University of Sao Carlos. The inoculated medium was transferred to adapted chamber (25 °C; light, 4000 lux). The algae were grown in Algae Media according to ABNT (2018) protocol. All the flasks, instruments and medium solution used through the experiments were previously sterilized (at 121 ºC; 15 minutes) in autoclave (Quimis, Q190M). More information about medium preparation is available in Appendix 2.

For EPS production, *R. subcapitata* were inoculated in Erlenmeyer flasks containing 2 L of Oligo Medium (pH 7.0-7.2) where the initial algal cell density was approximately 10^5 cells mL^{-1}. The algae were grown for 15 days (before the organisms reach the old phase) with aeration using pump air and constant illumination around 4000 lux at 25 ºC. After incubation, the algae solution obtained was centrifuged for 15 minutes at 3,500 rpm, the supernatant was carefully taken and the cells properly discarded (adding NaClO acting as a algicide). Guiven sequence to the procedure, the

EPS were collected using vacuum filtration system with nitrate cellulose filters (Sartorius, 0.45 µm) in order to remove possible remaining cells > 0.45 µm. The EPS solution was freeze-dried when necessary (e.g. characterization) or kept in solution (e.g. EPS-NPs interaction tests). For the last case, DOC concentration was determined by carbon analyzer (Analytik Jena, Multi N/C 3100) high temperature 850 °C oxidation. Potassium hydrogen phthalate (Synth P.A.) and sodium hydrogen carbonate (Qhemis P.A.) and sodium carbonate (Synth P.A.) were used as standard for calibration curve construction.

The Oligo medium type was chosen as Algae media since most common test medium on the guidelines contains ethylene tetra-acetic acid (EDTA), a chelating agent that might influence on nanomaterial behavior (Koukal *et al.*, 2007). Hund-Rinke et al. (2016) have suggested the use of modified medium in order to omit EDTA effect, which may be advantageous when investigating the toxicity of metal NMs, particularly in the presence of natural organic material.

3.2.1.1. Aquatic humic substances extraction

The AHS were collected in the Sorocabinha river, city of Iguape, Brazil (Latitude: 24°41′59″S, Longitude: 47°33′05″W). The AHS was obtained by chromatography separation using the resin Superlite™ DAX-8 (Sigma Aldrich) following recommended literature (International Humic Substances Society, IHSS; (Thurman and Malcolm, 1981). Briefly, the river water collected was acidified to a pH 2 and the humic material was retained in the resin when the water passed through the XAD-8 column in a slow stream. The AHS adsorbed was eluted with Sodium Hydroxide (P.A. Dinâmica) 0.1 $molL^{-1}$. The humic substances solution extracted were adjusted until pH 5.8, according to in situ measurement.

Characterization techniques were performed in order to evaluate the kinetic transformations of both NPs (1-100 μgL^{-1}) dispersed in two different medium (AM and DM), in absence and presence of both NOM previous produced (AHS and EPS). Two AgNPs commercially available with different manufacturing coatings were used: one single bond, such as citrate (AgCit) and neutral polymer such as PEG (AgPEG). The particles were acquired from PlasmaChem (http://www.plasmachem.com). The

physicochemical conditions that NPs exhibited were selected for their relevance to the organizations in the study:

(i) pH = 6.8 – 8.0;

(ii) sodium and/or potassium concentration = 2×10^{-4} - 2×10^{-3} M;

(iii) calcium concentration = 1×10^{-4} - 4×10^{-4} M;

(iv) magnesium concentration = 1×10^{-4} - 6×10^{-4} M;

(v) Ag NPs concentration: 1 μgL^{-1} - 100 μgL^{-1} of total Ag;

(vi) AHS concentration: 1 - 10 mgL^{-1};

(vii) EPS concentration: the same value produced by algae 1 º to 4 º day (time of expose of experiments). 1- 5 mgL^{-1};

The behavior of each NPs was analysed at specific exposition time (t_{exp}) according to bioassays protocols where algae. Due to the fact that time was considered a critical parameter in the characterization of changes of NPs and must be carefully controlled, the NPs were exposed to the physicochemical conditions described above for 4 to 7-8 days according to the time to be used for exposure experiments.

2.4.1 Charge density by Electrophoretic Mobility

Complementary electrophoretic mobility measurements were also proceeded in order to determine the charge density of NPs. The charge density is considered a fundamental property of colloids, and colloidal suspensions stability are considered critically depends on the amount of charge developed at the interfaces between the particles and its solvent, which will have a major impact on its conformation, mechanical and chemical stability. This complete characterization of the particle surface will assess the nature of the links between the manufactured coatings and the core of Ag NPs, establish relationships between the effectiveness of these manufactured coatings, whether simple ligands or neutral polymers, and the possible transformations of their own NPs Ag when exposed to environmentally relevant medium. In order to see the charge density of interactions EPM were measured adding 5 mL of Algae medium (AM) and/or Daphnia medium (DM) in the presence and absence of organic matter in terms of DOC 1 and 10 mg L^{-1} of HS and 1 and 5 mg L^{-1} of EPS, in the presence and absence of 2 mg L^{-1} Ag-Cit and Ag-PEG in terms of Ag

concentration. Each solution test previously described was tested in a pH range between 3 and 8. For pH adjustment, HNO_3 0.1 mol L^{-1} and NaOH 0.01 mol L^{-1} were added when necessary. After the adjustment NPs were added and the samples were left overnight and then the EPM meausements were carried out.

3.2.2. Speciation modeling calculation

The species distribution for different medium was performed for Ag^+ (NO_3) treatments using Minteq computing modeling. MinteQ calculates the concentration of various chemical species at chemical equilibrium based on the equilibrium formation constants for most inorganic species encountered in natural waters under standard conditions. Chemical speciation calculations for Ag+ and organic matter model (NICA-DONNAN) were also carried out. The composition of both freshwater solutions are following described. For algae medium exposure solution (Table 3. 1), ABNT (2018) was adopted as a protocol. In the case of Daphnia medium exposure solution (Table 3. 2), soft reconstituted water (hardness) was adopted (Abnt, 2016).

Adsorption of $AgNO_3$ solution (100 μgL^{-1}) in ultrapure water, daphnia media and algae media in the absence and presence of AHS 1 and 10 mgL^{-1} were also performed in order to see the difference of species due to the different media comparing to speciation by Minteq.

3.2.3. Dissolution by Centrifugation using ultrafiltration systems (CU)

The dissolution were assessed using ultrafiltration system (3 kDa pore Amicon, Millipore) and centrifugation technique. For that, 20 mL of test solution were prepared in polypropylene 50 mL falcon tubes for each condition by the time (t_{exp} = 0, 72 h and 96, 360 h, 720 h for AM and 0, 72 h and 96, 192 h, 360 h, 720 h for DM. In terms of DOC, AHS (1 and 10 mg L^{-1}), EPS (1 and 5 mg L^{-1}) concentration were also tested. Both AgCit and AgPEG were exposed to all conditions described above using two environmental relevant concentration, in terms of Ag (1 and 100 µg L^{-1}) (Conama, 2005; 2011).

All test solution were filtered using 3 kDa molecular weight membranes different exposition times (Figure 3. 1). The effective separation of the soluble portion and NPs

species can be a challenge, especially when using coatings with a metal complexing capacity of interest. For this specific case, the filtered portion corresponds to the free portion of Ag (Ag^+) plus all the small Ag complexes which have a correspondent size lower than the membrane pore, or 1-2 nm.

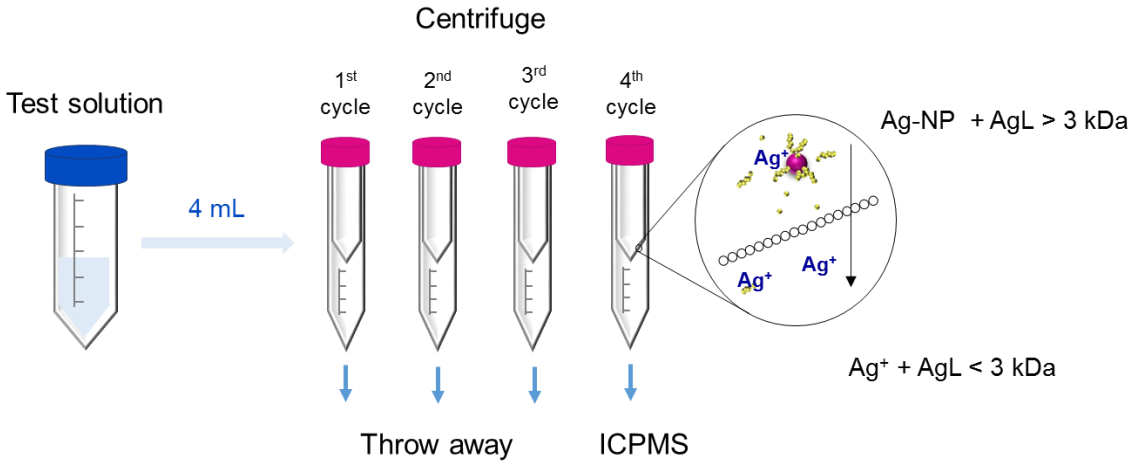


Figure 3. 1 - Schematic steps for ultrafiltration by centrifugation process.

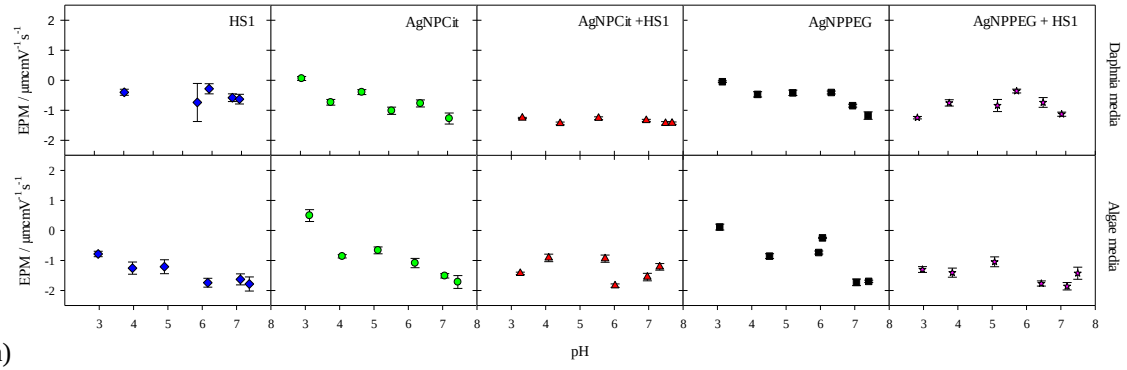
For the filtration process, 4 mL aliquots were taken and centrifuged during 35 minutes and 4,000 rpm. The filtered portion was discharged and 4 mL of test solution aliquot from the same flask tube was taken, centrifuged and again thrown away. This process was carried out 4 times (in total) and after the 4th cycle filtered portion was taken for analysis in High Resolution-Inductive Coupled Plasma Mass Spectrometer (HR-ICP-MS) (Element, ThermoScientific). The protocol containing more details about analyses procedures are disposed in the Appendix 3. After the data has been acquired from HR ICP-MS. UFREASI software was used for the treatment data and the detailed description with logic and mathematical manipulation behind is described in (Tharaud *et al.*, 2015).

3.3. Results and Discussion

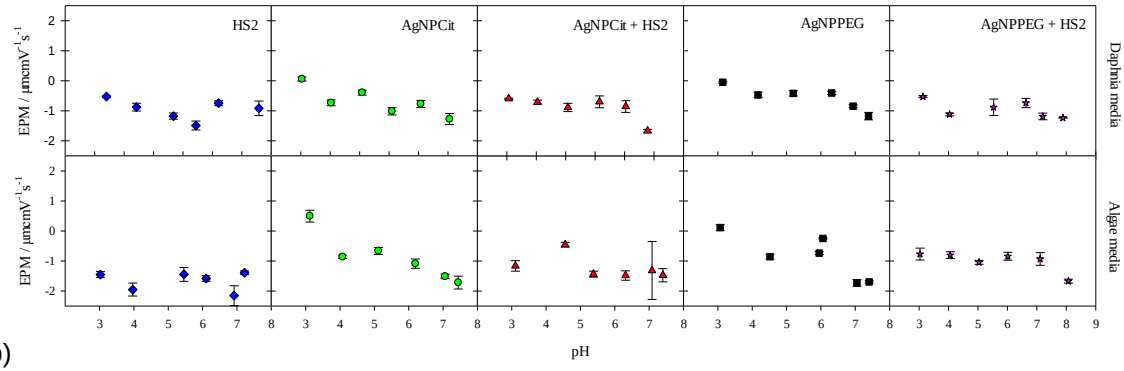
3.3.1. Charge density

The difference in the measured electrophoretic mobility as function of pH for the two silver nanoparticles is observed in Figure 3. 2. Generally, the EPM of AgNPs when in the presence of organic particles trend to be slightly negative. Observing the values

of EPM for 1 ppm of AHS (HS1) in the Figure 3. 2, more neutral EPM in Daphnia medium is observed comparing to Algae medium. Changes in solution pH (3-8) have more influence in EPM of both Cit and PEG capped AgNPs (Figure 3. 2) when there is the presence of HS1. In other words, when they are present, the EPM is slightly negative and more constant in all pH range. For the same medium, the two different capping agents for nanoparticles formation, generally presented the same EPM. Observing the Figure 3. 2b, 10 ppm of AHS (HS2) have same behavior of HS1, except for EPM of only HS2 that trends to be slightly more negative than HS1.



a)



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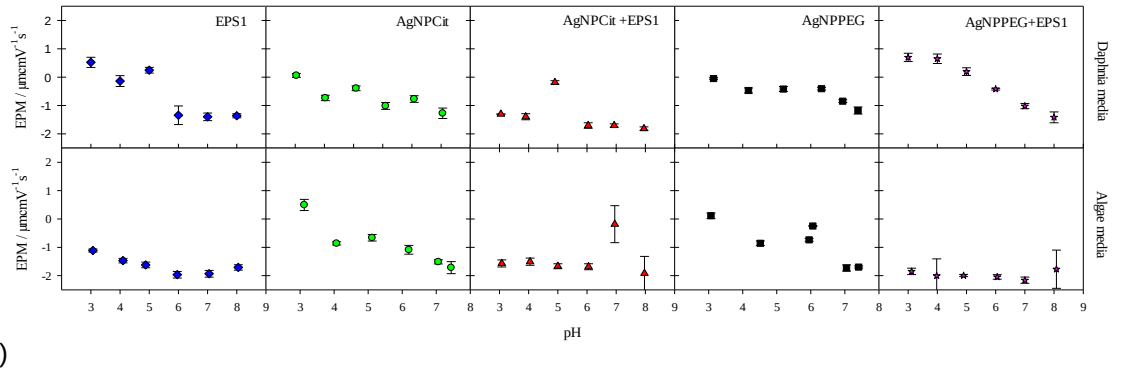
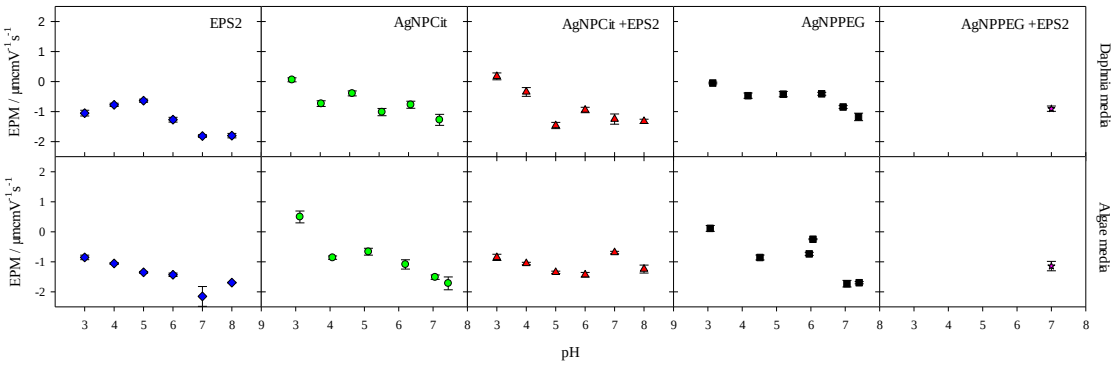


c)



d)

Figure 3. 2 - EPM measurements for a) HS1 = 1 mgL-1; b) HS2 = 1 mgL-1; c) EPS1 = 1 mgL-1; d) EPS2 = 5 mgL-1. (◇) AHS = 10 ppm, (○) AgCit = 2 ppm, (△) AgCit = 2 ppm + AHS, (□) AgPEG = 2 ppm, (✯) AgPEG = 2 ppm + SHA = 10 ppm, (◆) AHS = 10 ppm, (●) AgCit = 2 ppm, (▲) AgCit = 2 ppm + AHS, (■) AgPEG = 2 ppm, (★) AgPEG = 2 ppm + AHS = 10 ppm.

The presence EPS at 1 ppm and 5 ppm, EPS1 and EPS2, respectively, show different EPM depending on the exposed medium. Dispersed into Algae medium the EPS have no EPM changes with variation pH solution (3-8) while in Daphnia medium, differences in the measured electrophoretic mobility as a function of pH is observed. In presence of EPS1 (Figure 3. 2c), the difference of EPM observed between citrate and PEG capping agent including the different medium exposure show a strong effect of capping agent and solution.

The more negative EPM of AHS in both media is expected since they are usually negative for all pH range. Colloidal suspensions stability critically depends on the amount of charge developed at the interfaces between the particles and its solvent, which will have a major impact on its conformation, mechanical and chemical stability. Knowing particle surface is possible to assess the nature of the links between the manufactured coatings and the core of Ag NPs, establish relationships between the effectiveness of these manufactured coatings, whether simple ligands or neutral polymers, and the possible transformations of their own NPs Ag when exposed to environmentally relevant means.

According to literature, electrophoretic mobility measurements is normally taken as an indicator of surface charge. For a variety of different nanoparticles, the pH reveals a classic curve of increasingly negative electrophoretic mobility as solution pH becomes more basic (Jonathan *et al.*, 2007). Charge density can be influenced by ionic strength while mobility approaches zero with increasing ionic strength. In addition, the difference of EPM observed between citrate and PEG capping agent including the different medium exposure show a strong effect illustrates the importance of capping

agents when evaluating surface charging properties of nanoparticles. The results generated by Minteq tool calculation have showed values of ionic strength (IS) for both Daphnia and Algae media 1.369 and 0.1418 mol L^{-1}, respectively (pH 7.29 and 7.78). These results show considerable difference between the exposure solutions in terms of ionic strength and, in addition, that the composition of exposure medium could be a factor of influence in the EPM values obtained. Algae medium has higher IS than Daphnia medium, however in terms of divalent cations contents (Ca^{2+} and Mg^{2+}), Daphnia medium is the more concentrated. The literature has reported that divalent species have the ability of assist the formation of complexes between organic matter and NPs, resulting in aggregates. Once NOM (negatively charged) bound to the NPs, it can be neutralized by divalent cations, diminishing the EPM and contributing for NPs aggregation (Grillo *et al.*, 2015). The influence of pH is also reported on literature, as the pH increases, the concentration of OH- increases thus allowing the OH^- to more effectively compete for surface sites which generates a negative surface charge in alkaline pH environments (Badawy *et al.*, 2010).

3.3.2. Dissolution effect

Figure 3. 3 and Figure 3. 4 show the dissolution results obtained in presence and absence of the two natural organic matter, HS and EPS for AgCit and Ag PEG, respectively. The filtered portion in the present work corresponds to the free portion Ag (Ag+) plus all the small Ag complexes which have a size lower than the membrane pore, or 1-2 nm. The dissolution of AgNPs pH 6 and 7 and in absence of AHS increases with time independently of the NPs concentration and the medium nature, mainly until 408 h (17 days) from which it decreases until 1 month of exposure. For the conditions where Ag is observed in the filtrate, the dissolution of the AgPEG NPs increases with time until 200-400 h, after what it decreases. Contrarily to the AgCit, the AgPEG when dispersed in Daphnia media undergoes more dissolution at pH 7 than at pH 6 or 8. However, in presence of the algae media more Ag concentration was obtained in the filtrate at the lowest pH, followed by pH 7 and finally pH 8.

At pH 8, this increase of dissolution with time is much less prominent for both NPs concentrations and medium. In presence of 10 ppm of AHS, and independently of the NP concentration or the medium, there is almost no Ag quantifiable in the

Centricon filtrate. When in presence of the lower humic substances concentration, i.e., 1 ppm, there is a slight increase of the NPs dissolution until approximately 200 h, from which point it decreases. The Ag concentration in the filtrate is not significant when in presence of the largest humic acid concentration (lower than 5 %), i.e., 10 ppm, even at the highest NP concentration.

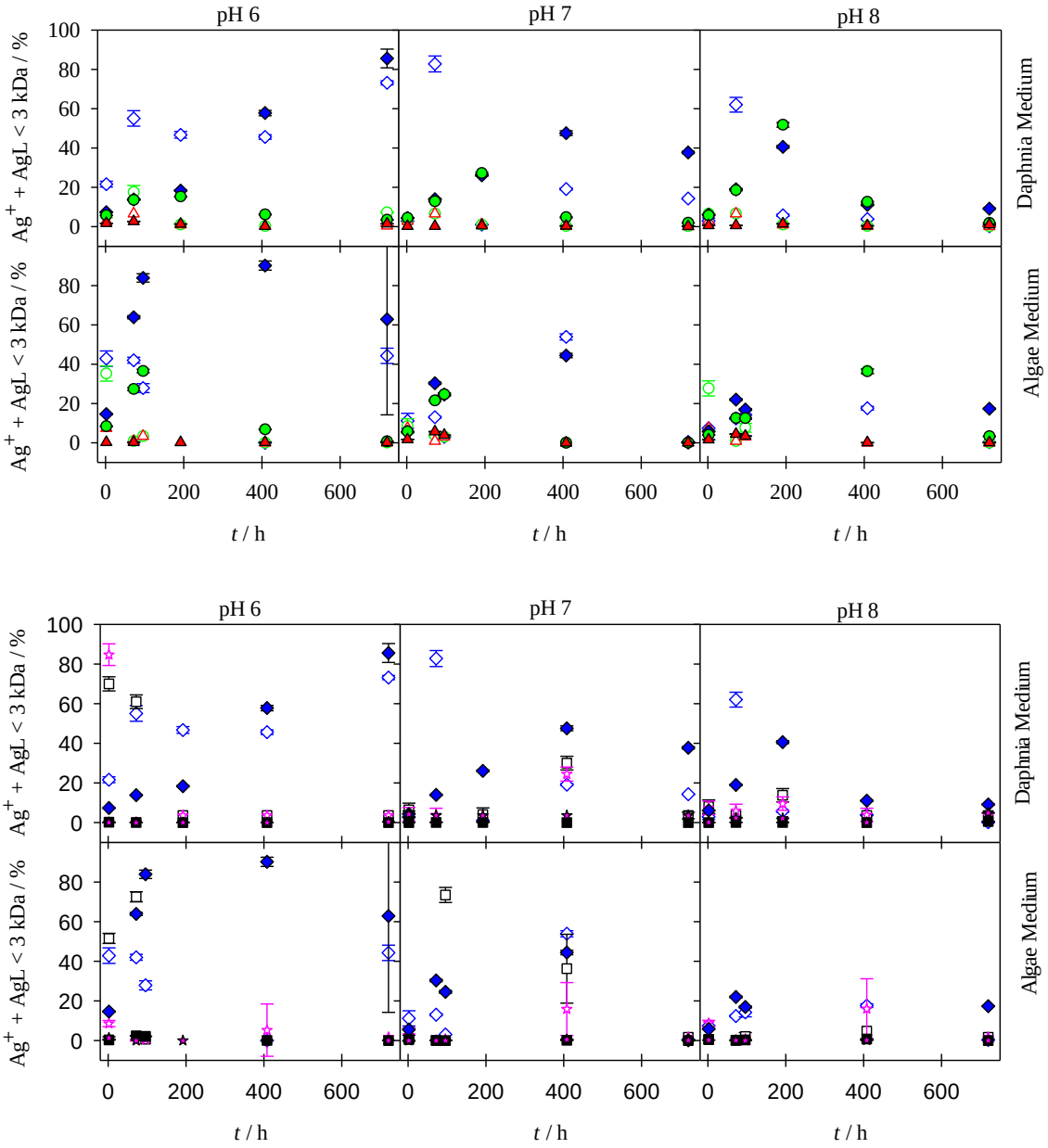


Figure 3. 3 - Percentage of free silver (Ag+) plus silver complexes (AgL) with lower sizes than the cut-off of the membrane pores (< 3 kDa) as a function of exposure time for AgCit when dispersed in Dapnhia and Algae medium at pH 6, 7 and 8 in absence and presence of organic matter. (◇) AgCit = 1 ppb, (□), (○) AgCit = 1 ppb + AHS = 1 ppm, (△) AgCit = 1 ppb + AHS = 10 ppm, (◆) AgCit = 100 ppb, (●) AgCit = 100 ppb + AHS = 1 ppm, (▲) AgCit = 100 ppb + AHS = 10 ppm. AgCit = 1 ppb + EPS = 1 ppm, (☆) AgCit = 1 ppb + EPS = 5 ppm, (■) AgCit = 100 ppb + EPS = 1 ppm, (★) AgCit = 100 ppb + EPS = 5 ppm. Error bars represent standard deviation of triplicates measurements.

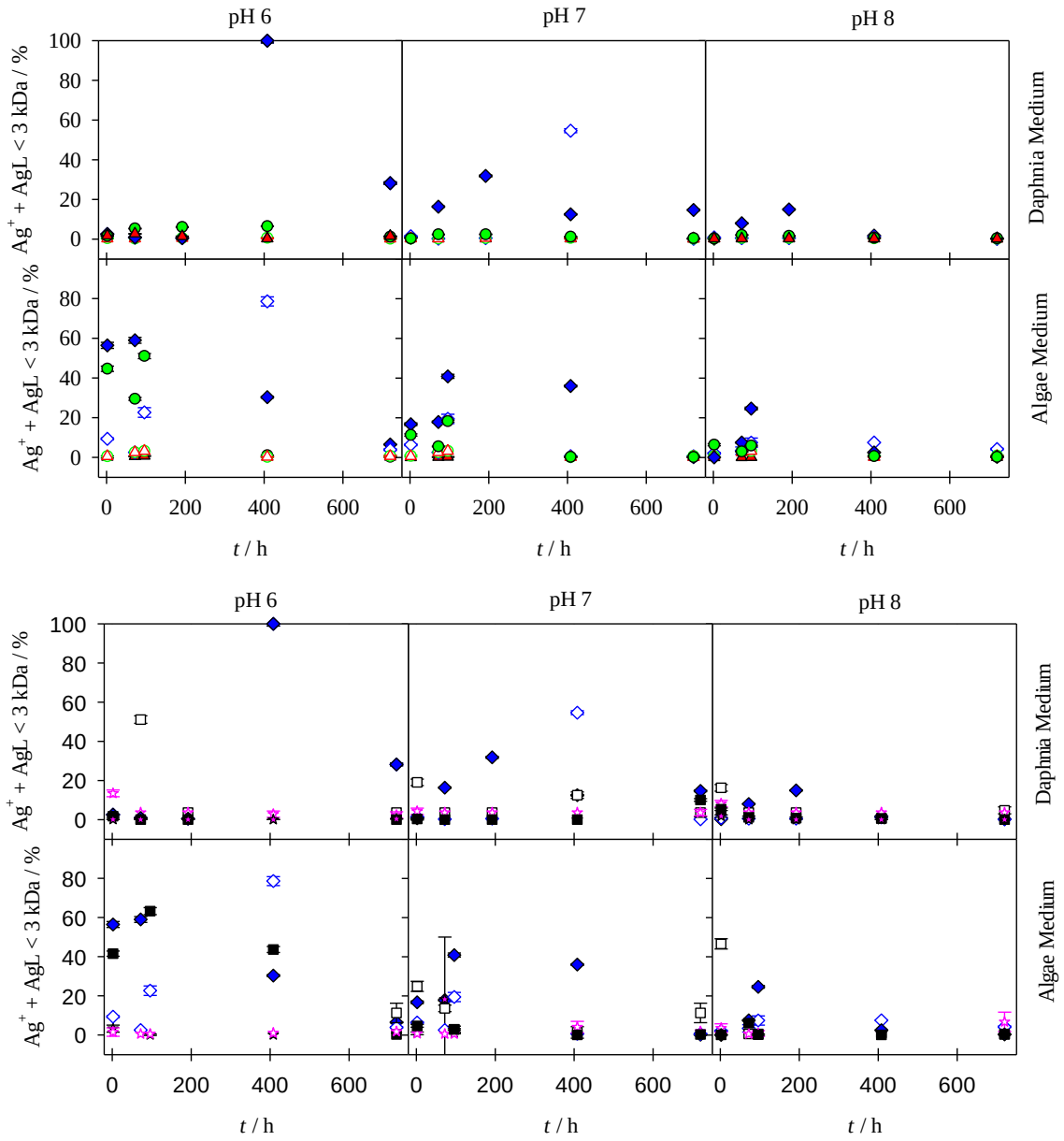


Figure 3. 4 - Percentage of free silver (Ag+) plus silver complexes (AgL) with lower sizes than the cut-off of the membrane pores (< 3 kDa) as a function of exposure time for AgPEG when dispersed in Dapnhia and Algae medium at pH 6, 7 and 8 in absence and presence of organic matter. (◇) AgPEG = 1 ppb, (○) AgPEG = 1 ppb + AHS = 1 ppm, (△) AgPEG= 1 ppb + AHS = 10 ppm, (◆) AgPEG = 100 ppb, (●) AgPEG = 100 ppb + AHS = 1 ppm, (▲) AgPEG = 100 ppb + AHS 10 ppm. (□) AgPEG = 1 ppb + EPS = 1 ppm, (☆) AgPEG = 1 ppb + EPS = 5 ppm, (■) AgPEG = 100 ppb + EPS = 1 ppm, (★) AgPEG = 100 ppb + EPS = 5 ppm. Error bars represent standard deviation of triplicates measurements.

In general, for all conditions and both media, the dissolution of the AgCit NPs decreases with the increase of pH. The highest dissolution is found at pH 6, whereas at pH 8 the lowest dissolution of the NPs is obtained. For the AgCit in absence and presence of 1 ppm of AHS and for both medium the highest dissolution was obtained for the highest concentration of the NPs, i.e., 100 ppb (with exception of the first time points for the Daphnia media in absence of AHS). When in presence of the highest

AHS concentration there is no effect of the NPs concentration since there is no significant Ag concentration in the filtrate. Higher AgPEG dissolution is obtained when the particles are dispersed in the algae media than in the Daphnia media. Furthermore, when AgNP PEG is dispersed in Algae medium, the dissolution is reduced, resulting in higher amounts of Ag dissolved in comparison with Daphnia medium. This is more visible at pH 6.

3.3.3. Organic matter effect

The presence of the AHS changes the dissolution rate and profile of the NPs independently of the media and pH. The presence of even low AHS concentration (1 mgL^{-1}) reduces drastically the dissolution of the NPs, whereas increasing the AHS concentration 10 results in not significant concentration of Ag in the centricons filtrate. Whereas at pH 6 the algae media seems to be more favorable to provokes dissolution, at pH 8 more Ag is found in the filtrates when the NPs are dispersed in the Daphnia media.

The dissolution is generally higher for the highest AgPEG concentration (100 μgL^{-1}), even when in presence of the humic substance. In the Daphnia media the presence of lowest AHS concentration results in almost no significant Ag amounts on the centricons filtrates. In the algae media, and when in presence of the highest concentration of NPs, the addition of the 1 mgL^{-1} of AHS, it still remain some dissolution of the NPs at pH 6 and 7. However, when in presence of the highest AHS concentration no significant Ag concentration is measured in the filtrates.

The presence of EPS, also as observed for AHS (Figure 3. 3), changes the dissolution rate/ profile of the AgCit. When in presence of 1 ppm of exopolysaccharides, there is a slight increase of dissolution until 400 h, followed by a notable dissolution decrease. In presence of 5 ppm of EPS and highest AgCit concentration, independently of the medium, almost no Ag is detected in the filtrate were for all exposition time. For the lowest AgCit concentration, it is possible to notice remaining Ag in the filtrate until 400 h. According to these data, it is possible to identify an influence of organic matter concentration on the AgCit dissolution profile.

Almost no influence of EPS, to minimize the dissolution, is observed when AgCit is exposed to 1ppm of EPS at pH 6 is observed in both medium. For pH 7 and 8 and

small amounts of AgCit, it is possible to notice a slight increase of dissolution in the algae medium over daphnia medium. In the case PEG as previously mentioned the nanoparticles shows more stable, due to the low Ag dissolved concentration amounts (Figure 3. 4). Nevertheless, when dissolution occurs, it is possible to notice a reduction onto dissolution due to the presence of EPS. For the highest concentration of silver, the contents of EPS have influenced in the decrease of dissolution rate.

3.3.4. Speciation

The speciation of Ag using Nica-Donnan model in Algae medium (Table 3. 3) shows that the highest content of silver species was found in AgCl(aq) form, independently of the NOM amount. The table of Ag species for Daphnia medium (Table 3. 4), shows that Ag free ion represent around 21 % of the total concentration of Ag while the most abundant specie should be AgCl(aq), representing around 62 % of the total.

Table 3. 3 - Speciation of Ag in Algae medium in presence of organic matter (NICA-DONNAN model).

Species name		Ag^{+}	$AgCl_{(aq)}$	$AgCl_2^{-}$	$AgCl_3^{-2}$	$AgSO4^{-}$	$AgNH3^{+}$	$AgNO_{3(aq)}$
Exposure condition		% of total concentration						
C_{Ag} / M	C_{NOM} / mgL^{-1}							
9.35E-09	-	10	53	32	1	2	2	4
	1	10	53	32	1	2	2	4
	10	10	53	32	1	2	2	4
9.35E-07	-	10	53	32	1	2	2	4
	1	10	53	32	1	2	2	4
	10	10	53	32	1	2	2	4

Table 3. 4 - Speciation of Ag in Daphnia medium in presence of organic matter (NICA-DONNAN model).

Species name		Ag^{+1}	$AgCl_{(aq)}$	$AgCl_2^{-}$	$AgSO_4^{-}$
Exposure condition		% of total concentration			
C_{Ag} / M	C_{NOM} / mgL^{-1}				
9.35E-09	-	21	62	14	3
	1	21	62	14	3
	10	21	62	14	3
9.35E-07	-	21	62	14	3
	1	21	62	14	3

	10	212	62	14	3

The adsorption results (Figure 3. 5) show the Ag^+ presented different behavior due to solution composition. For ultrapure water, in principle, no variation in terms of total concentration is observed during the experiment. However, when the silver was exposed to daphnia and algae medium, there is a reduction in terms of total concentration with time. Comparing to the species distribution, it's possible to relate to the AgCl formation which it is presented 53 and 62 % for Algae and Daphnia medium, respectively. In contrast with the modeling results, the presence of organic matter reduces the Ag^+ with the increasing of carbon amount.

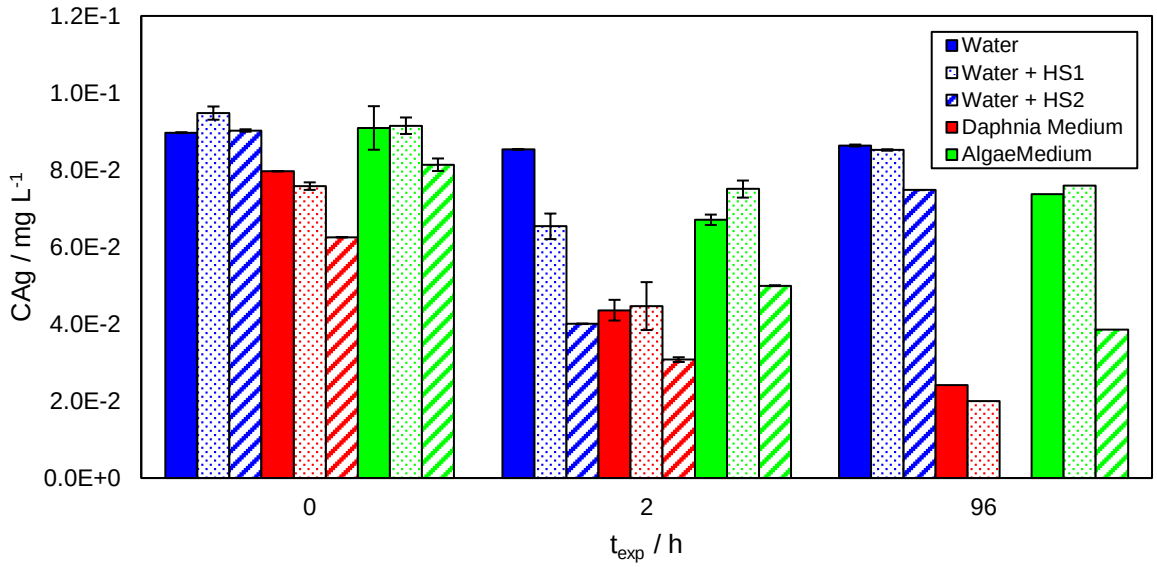


Figure 3. 5- Adsorption of AgNO3 solution (100 µgL-1) in ultrapure water (blue), daphnia media (red) and algae media (green) in the absence (solid color) and presence of AHS 1 (dots) and 10 (stripes) mgL-1.

The model suggests the low affinity of silver due to the presence of NOM. (Sikora and Stevenson, 1988) have reported results of low affinity of Ag ions in the presence of humic substances only taking into account phenolic and carboxylic groups. However, according to the authors, the presence of N and S groups improve the interaction between silver and HS. According to the characterization performed in the Chapter 2, EPS has higher nitrogen amounts than AHS also evidenced by FT-IR analyses, showing the presence of amine groups. Then, the probably interaction between NOM and AgNP can be due to the presence of nitrogen groups.

According to the results observed, either absence and presence of the lowest and highest AHS concentration and for the range of Ag NPs concentration, the AgCit

NPs undergoes more dissolution than AgPEG. Liu and Hurt (2010) (Liu and Hurt, 2010) have showed that dissolution of silver nanoparticles capped with citrate dispersed in deionized water is dependent on the pH and dissolved oxygen.

The decrease of dissolution in the presence of both organic matter, AHS and EPS, is observed. One of the possible reasons for this behavior can be attributed to the adsorption of nanoparticles by natural organic matter. These natural entities can bind to the surface of the NPs forming a coating around these and affecting its physical-chemical and biological identity, and thus affecting its surface reactivity and interactions with cells and organisms. Thereby, NOM can arrange to NPs electrostatic or steric stabilization, or result in flocculation (Baalousha *et al.*, 2008; Domingos *et al.*, 2009). Baalousha et al. (2013) reveal that the complex formation depends on both the type of nanoparticle and the composition of organic matter. The composition of both organic matter studied by FT-IR spectroscopy shows the high potential of interactions with organic and inorganic substances due to the presence of binding sites (e.g. phenol and carboxylic groups, polysaccharides, amine and nitrogen groups). Evidences of EPS binding ability was reported by Liu and Fang (2002). The authors have characterized electrostatic binding sites of EPS from microbial communities comprising 10.88 and 16.44 mmol g^{-1} EPS of binding sites. Based on these results, the authors speculate that EPS are likely to play a more critical role in metal absorption than cell surface of the bacteria.

Due to the different exposure media and taking to account the element composition of both media according to the Table 3. 1 and Table 3. 2, both media may help the interaction between NOM and the ENPs by cation bridging interaction mechanism. In that sense, divalent metal ion (e.g., Ca^{2+}, Mg^{2+}) are capable to bridge functional groups of the nanomaterial and the organic material, thus facilitating the sorption (Dong and Lo, 2013). Another possible effect of the interaction AgNP-NOM is reported by Diegoli et al.(Diegoli *et al.*, 2008). The authors have showed that humic acid was able to substitute citrate capped on gold nanoparticles and retard aggregation due to steric stabilization. The relevance in the studies of the dissolution rate of nanomaterials lies in the fact that several studies in NMs toxicity had suggested that dissolution of Ag^{+} ions from AgNPs is the main mechanism of the antibacterial activity of AgNPs (Chaloupka *et al.*, 2010).

3.4. Conclusions

In resume, condering the electrostatically interactions between silver nanoparticles and organic matter, it was possible to identify the that the charge can be related both pH and ions present in the medium composition. The amounts of DOM and particle concentration affect the dissolution that also depends of the coating type (Citrate or Peg). Furthermore, organic matter have strong influence on avoiding AgNP dissolution and the presence of both organic matter studied (EPS and AHS), provokes a NP stabilization in the exposed medium. Exposure medium (AM and DM) also affects the AgNP transformations showing the importance of studies taking into account environmentally relevant matrices. The investigation performed in the present work demonstrates the influence of both organic matter toward the fate of silver nanomaterials. This approach will allow a breakthrough in the understanding of environmental and biological fate of nanomaterials and could certainly have a considerable impact on regulatory guidelines.

CHAPTER 4: INFLUENCE OF NATURAL ORGANIC MATTER IN THE ECOTOXICOLOGY AND BIOACCUMULATION OF CAPPED SILVER NANOPARTICLES USING RAPHIDOCELIS SUBCAPITATA

Abstract

Despite the growing interest and use of nanomaterials (NMs), the gathered knowledge on their environmental consequences is still scarce since most of the studies do not even determine the effects of the presence of manufactured coatings. Generally, NMs add ionic or polymeric coatings to improve their mobility and stabilization in terms of size. The present work aims evaluate the toxic effects of manufactured coated AgNPs in absence and presence of humic substances (HS) on the algae *Raphidocelis subcapitata* and silver bioaccumulation in order to explore the impact of natural molecules on their environmental risk of these NMs in the aquatic environment. The algae were exposed to AgNPs with manufactured coatings in the absence and presence of HS. In parallel to the NPs exposures, silver bioaccumulation were also quantified. Growth inhibition of algal growth was determined by the biomass produced after the tests using the count of the initial and final cell numbers. The toxic effect of silver ions did not changed with the presence of natural organic matter. The difference of capping agents as well as nanoparticle concentration shows different toxicological responses by observing a AgPeg toxicity compared to the same Ag concentration using Citrate which did not presented toxic effect. For bioaccumulation, the presence of natural organic matter enhanced the silver amount in the algae probably due to surface bound of HS-Ag. Finally, the presence of NOM influences in the silver bioaccumulation, where in the case of AgCit, there is a decrease of silver accumulated in the presence of humic material.

Keywords: nanosilver; ecotoxicity; bioaccumulation; humic substances.

4.1 Introduction

Manufactured nanoparticles (NPs) can interact with abiotic molecules in the environment, yielding a transformed NP, with new distinct properties. Despite the

intense research being done, the gathered knowledge on their environmental interactions is still contradictory. In the case of silver nanoparticles, dissolution has been reported as the main mechanism of toxicity of nanoparticles (Ratte, 1999). It is fully recognized that free silver ion (Ag+) is highly toxic to a wide variety of organisms including inhibitory effect to bacteria.

Algae are condidered the primary producers of biological organic matter (biomass) in water. They use the light energy and store it as chemical energy. In the absence of sunlight, however, the same chemical energy are consumed by them for metabolic needs. In general, they are microscopic organisms that subsist on inorganic nutrients and produce organic matter from carbon dioxide by photosynthesis. Commonly known as green algae, *Chlorophyta* are responsible for most of the primary productivity in freshwaters. The photosynthesis process fixes carbon dioxide and inorganic carbon from dissolved carbonate species as organic matter, thus providing the basis of the food chain for the other organisms in the system. The production of moderate amounts of biomass is beneficial to the other organisms in the aquatic system (Manahan, 2005). *Raphidocelis subcapitata* is a single-cell algae considered easy to grow due to its rapid growth and a representative organism of freshwater environments (Hoffman *et al.*, 1995). Bioaccumulation is the uptake of substances via body surfaces (bioconcentration) and through food uptake (biomagnification) (RATTE, 1999).

Humic substances is a complex and heterogeneous portion of dissolved organic matter (DOM) present in freshwater environments (Rocha and Rosa, 2003). In general lines, DOM has many functions such as the ability to modify the processes in ecological systems ref. Given this context, the present work aims at evaluating: i) the toxic effects of manufactured coated AgNPs in absence and presence of humic substances (HS) on the algae *Raphidocelis subcapitata* and ii) silver bioaccumulation in order to explore the impact of natural molecules on their environmental risk of these materials in the aquatic environment.

4.2 Materials and Methods

4.2.1 Test organism and culture maintance: R. subcapitata

The experiments were performed based on the protocols established by United States Environmental Protection (USEPA) Agency, Organisation for Economic Co-operation and Development (OECD) and Brazilian standard protocols (ABNT) (Usepa, 2002; Oecd, 2011; Abnt, 2018). Cultures of *R. subcapitata* microalgae were carried out according to (Abnt, 2018). In addition to bioaccumulation and toxicity tests, these organisms were used as food in *D. similis* cultures. For the maintenance of *R. subcapitata* cultures, it was decided to use L.C. Oligo liquid culture medium also referenced as Algae Medium (AM), prepared from 7 solutions containing nutrients selected for algae development. Thus, Appendix 2 provides the preparation of each solution required for the composition of Oligo medium as well as the volume used to prepare 1 liter of culture medium.

4.2.2 Sensitivity test

Sensitivity tests were performed onto the organism *R. subcapitata* according to (Abnt, 2018). The tests evaluates the sensitivity performed throughout the year based on a known reference substance, in this case sodium chloride (NaCl). To this end, the EC_{50} was established through algal biomass produced. Five NaCl concentrations (0.4, 0.8, 1.6, 3.2, 6.4 gL^{-1}) were established based on the sensitivity values for the same organism by Santos, Vicensotti and Monteiro (2007). Table 4. 1 present the test solution preparation for those tests.

Table 4. 1 - Preparation of test solutions from a NaCl 128 stock solution gL-1.

Concentration (g L^{-1} NaCl)	**$Stock_{NaCl}$ (mL)**	**$Water_{ultrapure}$ (mL)**	**Oligo Medium (mL)**	**Inocule (mL)**
Controle	0	6,00	94	0,1-1,0
0,4	0,31	5,69	94	0,1-1,0
0,8	0,63	5,37	94	0,1-1,0
1,6	1,25	4,75	94	0,1-1,0
3,2	2,50	3,50	94	0,1-1,0

6,4	5,00	1,00	94	0,1-1,0

One week before the week preceding each test, Pre-culture preparation was performed. More details of this procedure is described in the Appendix 4. The cell concentration was determinatied using a Fuchs-Rosenthal chamber counting copled to a biological microscope (Figure 4. 1). The volume of the inoculum to be inserted (Equation 4. 1) as well the for the counting are following described (Equation 4. 2)

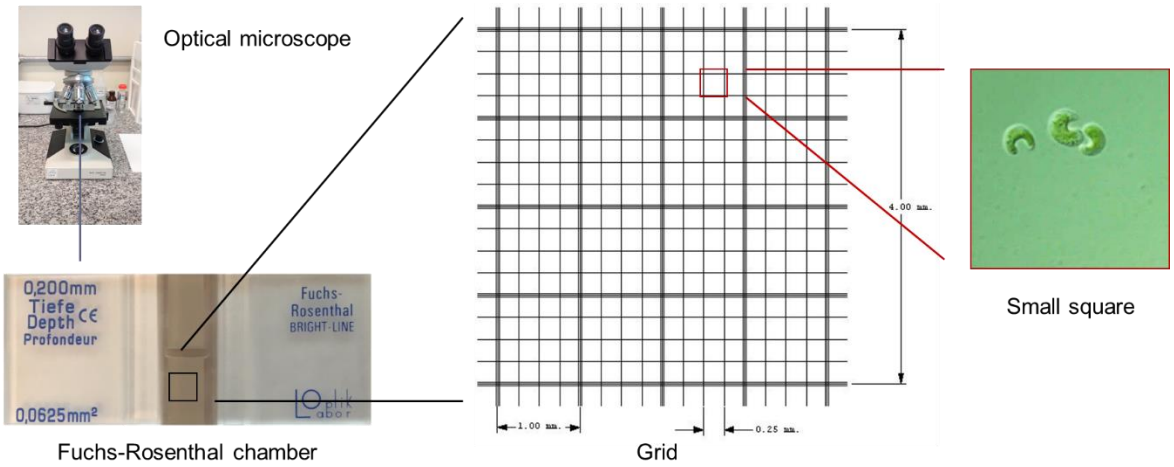


Figure 4. 1 - Fuchs-Rosenthal counting chamber for determination of cell concentration

$$V_i = \frac{C_i * V_f}{N}$$

Equation 4. 1 - Formula to determining the volume of algae to be inoculated for culture maximization.

Where:

Vi = inocule volume (mL)

Vf = solution volume (mL)

Ci = initial concentration (cells mL^{-1})

N = cells in suspension (cells mL^{-1})

$$C_f = \frac{N * 160.000}{Q}$$

Equation 4. 2 - Determination of algal solution concentration in Fuchs-Rosenthal chamber.

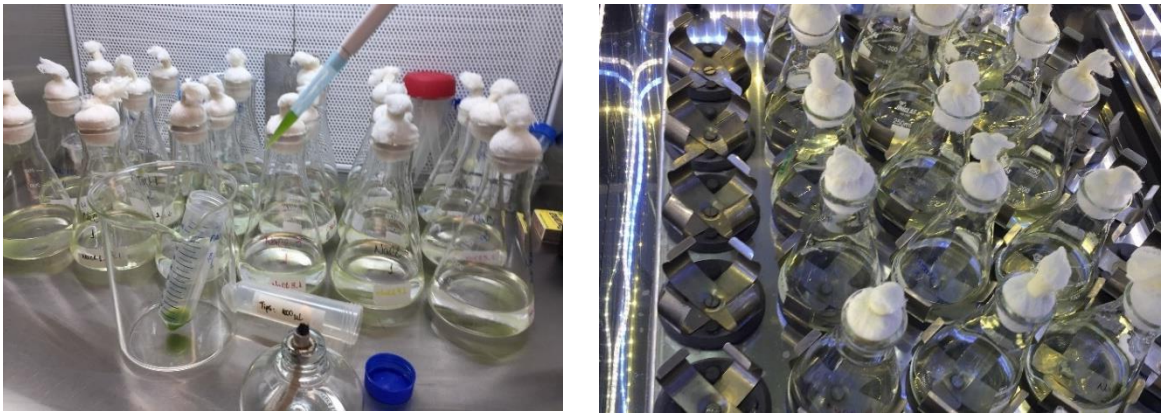
Where:

C_f = algal concentration (cells/mL)

N = number of cells counted.

Q = number of squares counted.

To initiate the tests, known volumes of algal inoculum were added to the solutions (Figure 4. 2a) to obtain an initial concentration between 10^4 and 10^5 cells mL^{-1}. In order to obtain the most accurate initial concentration possible for use in cell growth calculations, aliquots were taken at the initial exposure time. The samples were kept under agitation (Figure 4. 2b) and constant light for 72 hours and the flasks were randomly shifted every 24 hours (Ex. 24h, 48h, 72h). After completion of the assay, the test solution was fixed with lugol solution (80 mL for each 2 mL of solution) and cells were counted in a Fuchs Rosenthal chamber as described above. The counted values were recorded and at the end of the tests the concentration value corresponding to the EC_{50} obtained through statistical analysis by the ICpin software was generated.



(a) (b)

Figure 4. 2 - R. subcapitata: a) Algae inoculation to start testing and b) ongoing toxicity test.

In order to verify the possible toxicity of the extracted HS, toxicity tests were performed containing only Oligo Medium and HS 10 mg L^{-1} in terms of Organic Carbon, using the same procedures as previously used based on ABNT (2018). Likewise, tests were performed with both matrix solutions used during the synthesis process of the two nanoparticles studied.

4.2.3 $AgNO_3$ assay

Silver standard solution were prepared using silver nitrate powder firstly dried in a H_2SO_4 steam desiccator for 4 hours, 0.16 g of dry $AgNO_3$ dissolved in 1 liter of water. 1 mL of this solution contains 0.1 mg of Ag. The preparation was was taken from Morita (1968).

Assays with *R. subcapitata* microalgae were first performed using 5 concentrations within a range of 1 to 100 µg L^{-1} in terms of Ag (Ag^+) ions with the standard silver nitrate solution in order to obtain the EC_{50} value for silver ion. The second assay was performed using specific concentrations of 1 and 100 µg L^{-1} $AgNO_3$ (in Ag^+ terms) in the absence and presence of HS (1 and 10 mg L^{-1}).

Following the same procedure of item 4.2.2, each test solution was prepared in triplicate and for all tests performed a Control solution was prepared, containing only the nutrients necessary for microalgae growth without any contamination. The samples were kept under agitation and constant light in a Shaker incubator with shaking control (175 rpm) and temperature (25 ± 2 °C) for 96 hours, as shown in Figure 4. 2b, by changing positions of the test vessels at randomly. every 24 hours.

4.2.4 Silver nanoparticles toxicity tests

The microalgae *R. subcapitata* assays were performed using two concentrations, 1 and 100 µg L^{-1} in terms of Ag ions, of AgCit and AgPEG stock solutions in the presence and absence of natural organic matter 1 and 10. mg L^{-1} in terms of organic carbon (HS1 and HS2, respectively).

4.2.5 Statistical analysis

In order to identify eventual acute toxic effects were evaluated by comparing the algae growth was evaluated comparing the variance of control and test exposition by Fisher's Exact Test significant level ($p<0.05$) analyses using Toxstat 3.5 (West and Gulley, 1996)

4.2.6 Bioaccumulation Tests

Bioaccumulation assays were performed together with toxicity assays. Therefore, as shown in Figure 4. 3, at certain exposure times (0 h, 72 h and 96 h), 40 mL of test solution (in duplicate) was filtered using ester cellulose type filters (0.45 µm, Satorius) using a manual vacuum pump. In order to reduce possible contamination from the filters, they were left overnight in 5% (v / v) nitric acid solution. With the aid of pre-decontaminated plastic tweezers, the filters were extensively washed with autoclaved ultrapure water and then carefully arranged in the polysulfone (Nalgene) filtration system. After filtration of 40 mL of test solution, 5 mL of 0.1 M EDTA was added to remove any adsorbed materials on the cell walls. Then, with the aid of tweezers, the filters were carefully removed and arranged in falcon-like tubes and finally 0.3 mL of distilled HNO_3 (bioaccumulated) was added. The tubes were kept in a preheated oven at 85 °C until the filters were fully digested (about 12 hours). The filtered portion was called dissolved and a portion of the unfiltered test solution, called total, both being acidified using distilled nitric acid.

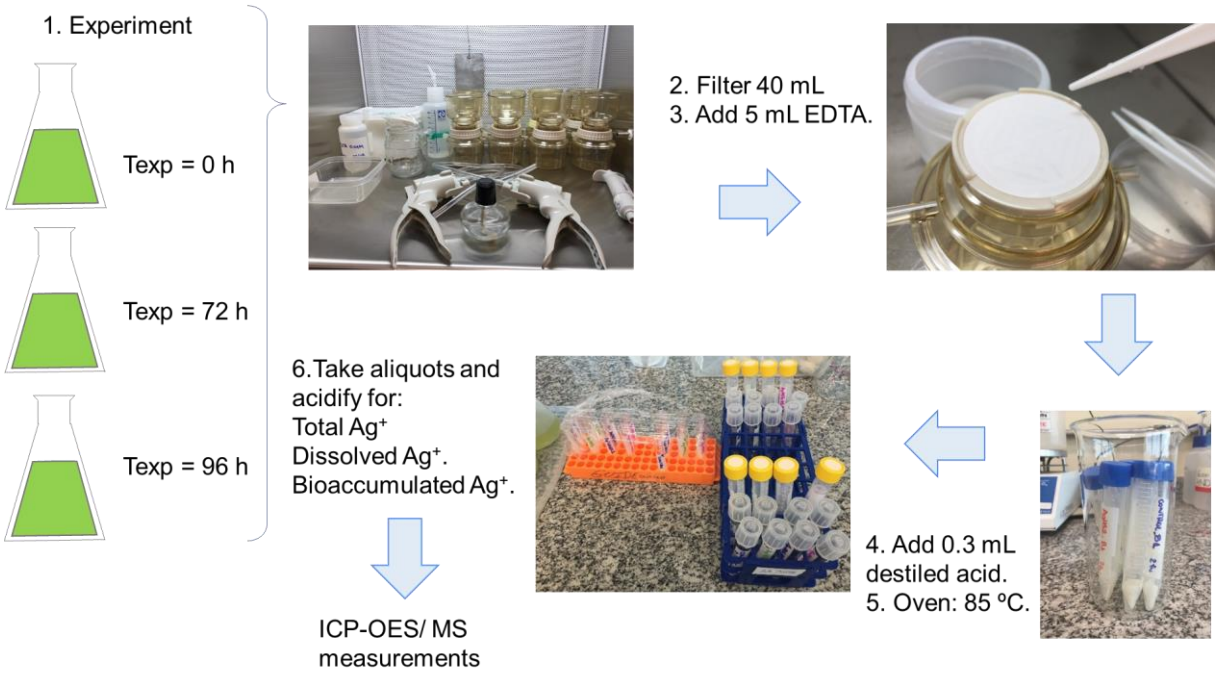


Figure 4. 3 - R subcapitata bioaccumulation assay schedule.

4.3 Results and discussion

4.3.1 AgNO3 bioassays

$AgNO_3$ EC_{50} results are shown in the Figure 4. 4. The test resulted in EC_{50} = 17.83 ± 1.18 μgL^{-1} in terms of Ag^+. Similar values were found by other authors (EC_{50} = 33.77 µg L^{-1}) performing tests using AgNPs (Ribeiro *et al.*, 2014).

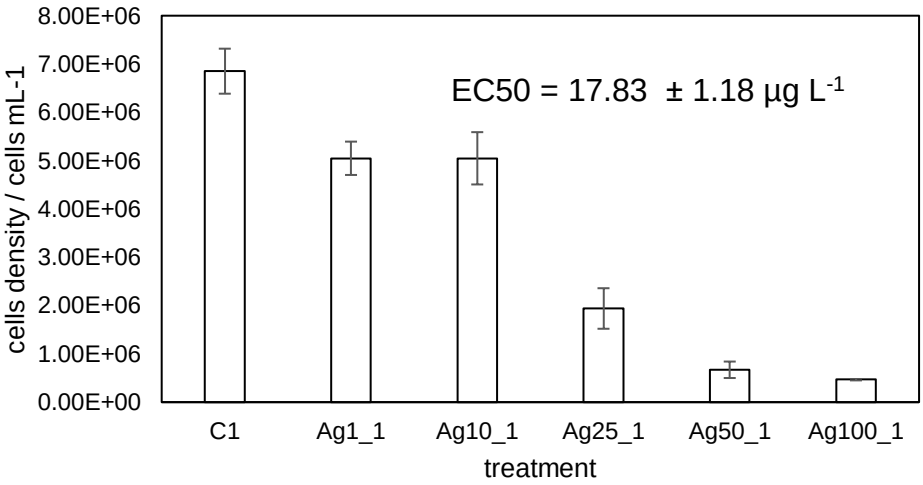


Figure 4. 4 - Toxic effect of AgNO3 on green algae R. subcapitata. Mean values ± SD (N = 3).

$AgNO_3$ toxicity results at concentrations 1 and 100 µg L^{-1} can be seen in the Figure 4. 5 and Figure 4. 6, respectively. At lowest concentrations (1 µg L^{-1}) the toxicity effects were minimal. The same is not true for the highest concentration, where the significant difference from the control is observed. In the presence of HS, a significant difference ($p < 0.05$) is also observed in relation to the Control even in the presence of HS in higher concentrations. The results show that the presence of natural organic matter did not prevent the toxicity effect for the silver ion.

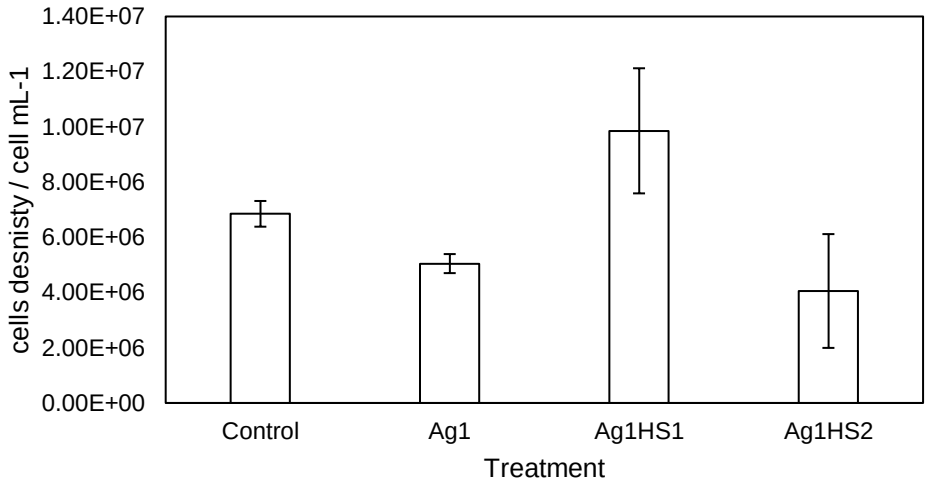


Figure 4. 5 - Toxic effect of R. subcapitata green algae in the presence of 1 µg L-1 AgNO3. Mean values ± SD (N = 3). HS1 = 1 mgL-1 HS; HS2 = 10 mg L-1 HS. * Statistically significant difference compared to control (p <0.05).

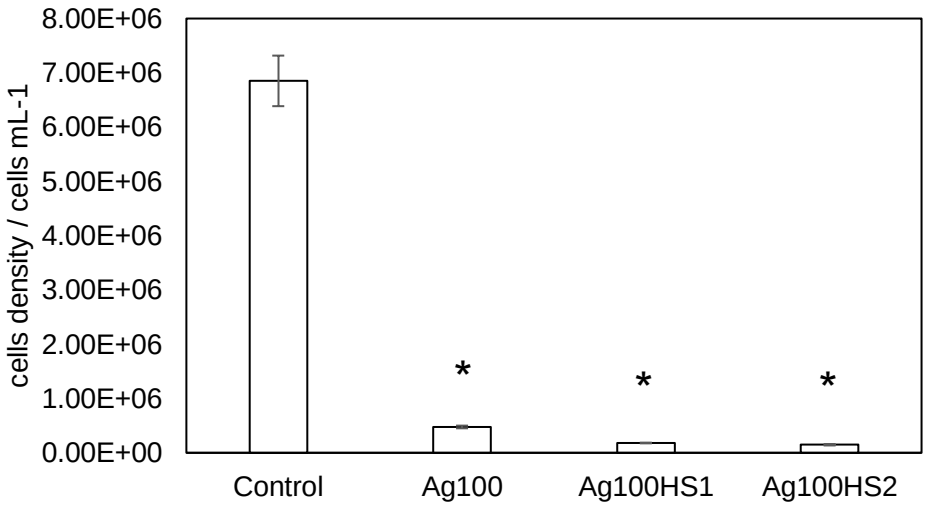


Figure 4. 6 - Toxic effect of green algae R. subcapitata in the presence of 100 µg L-1 AgNO3. Mean values ± SD (N = 3). HS1 = 1 mgL-1 HS; HS2 = 10 mg L-1 HS. * Statistically significant difference compared to control (p <0.05).

4.3.2 AgCit bioassays

The toxicity results of citrate-coated nanoparticles at both concentrations are shown in Figure 4. 7 and Figure 4. 8. For the lowest concentration, a significant difference with respect to control was observed when humic substances were present at the highest concentration. However, such an effect occurs in the form of stimulation in cell production, not inhibition of algal growth. For the higher concentration of citrate coated nanoparticles, both in the absence and presence of HS at both organic matter

concentrations, there is no toxicity effect. The same result was found by Angel et al. (2013) that performed bioassays using *P. subcapitata* and obtained higher values of IC_{50} of AgCit in the presence of humic material.

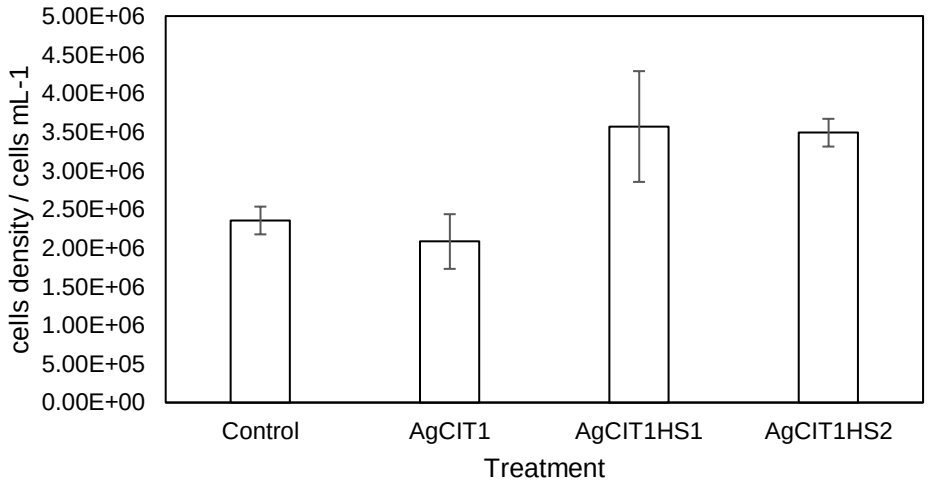


Figure 4. 7 - Toxic effect of R. subcapitata green algae in the presence of AgCit 1 µg L-1. Mean values ± SD (N = 3). HS1 = 1 mgL-1 HS; HS2 = 10 mg L-1 HS. * statistically significant difference compared to control (p <0.05).

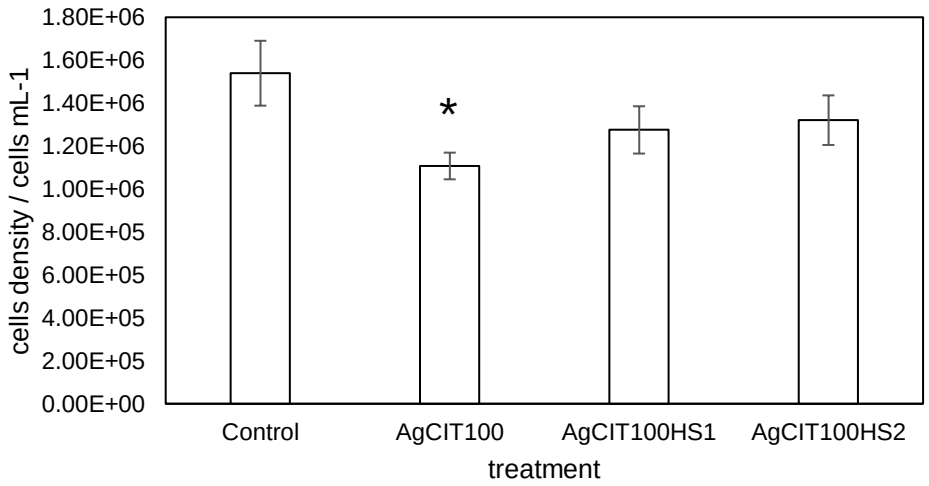


Figure 4. 8 - Toxic effect of R. subcapitata green algae in the presence of AgCit 100 µg L-1. Mean values ± SD (N = 3). HS1 = 1 mgL-1 HS; HS2 = 10 mg L-1 HS. * Statistically significant difference compared to control (p <0.05).

When comparing the toxicity results obtained with the algal dissolution results, a dissolution reduction ratio accompanied by a non-toxic effect in the presence of aquatic humic substances can be observed. One hypothesis for this behavior may be related to the stabilization that organic matter can provide to the coated NMs. According to the literature, natural entities bind to the surface of NPs forming a coating around them, affecting their surface reactivity and interactions with cells and

organisms. NOM can promote electrostatic or steric stabilization of NPs or result in flocculation (Baalousha *et al.*, 2008); It is worth remembering that the presence of EPS in the system may influence the stabilization of NMs, which is why there is no toxicity effect even in the absence of AHS.

4.3.3 AgPEG Assays

The toxicity results of polyethylene glycol coated nanoparticles are shown in Figure 4. 9 and Figure 4. 10. It is observed that there was no significant difference in all exposure conditions for the lowest concentration. However, the high value of the standard deviation in relation to the control should be observed, and influenced in the false negative identification for the toxic effect. When comparing toxicity data with dissolution experiments, there was no direct relationship between reduction of toxicity against nanoparticle dissolution.

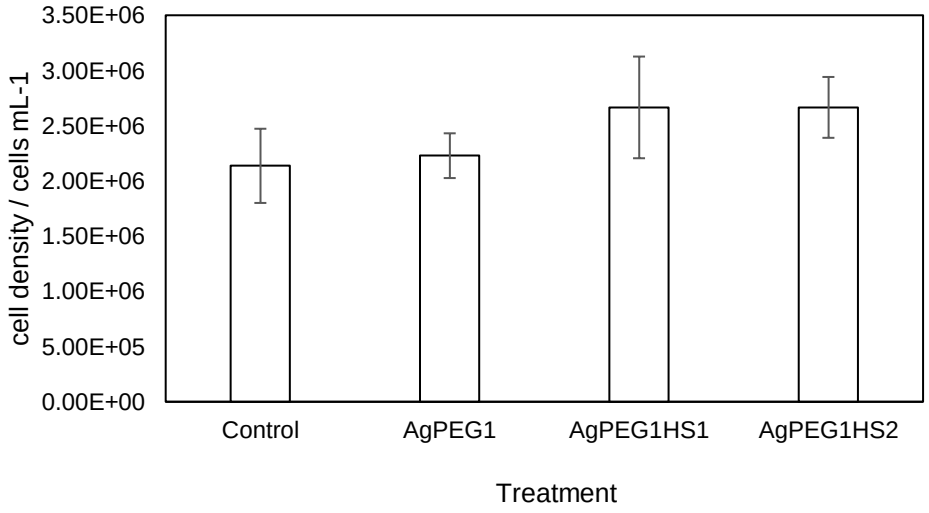


Figure 4. 9 - Toxic effect of R. subcapitata green algae in the presence of AgPEG 1µg L-1. Mean values ± SD (N = 3). HS1 = 1 mgL-1 HS; HS2 = 10 mg L-1 HS. * Statistically significant difference compared to control ($p < 0.05$).

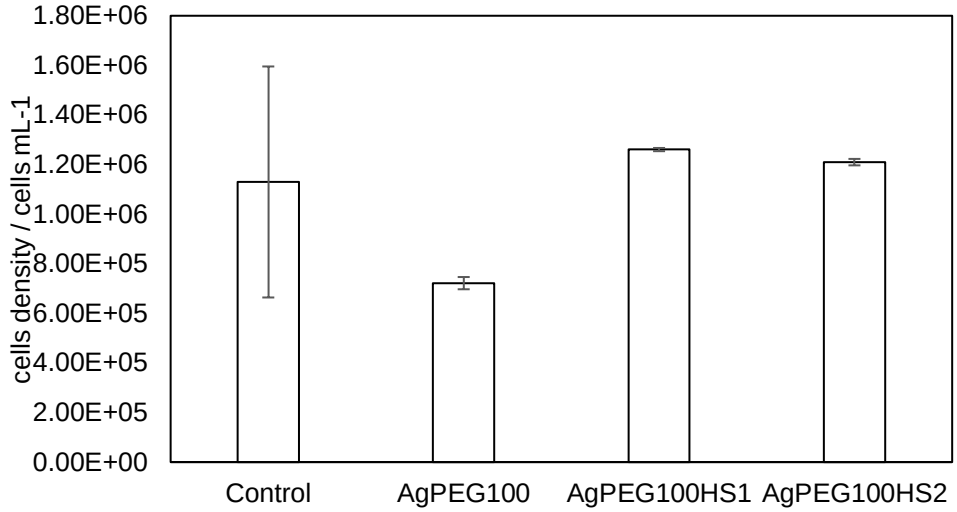


Figure 4. 10 - Toxic effect of green algae R. subcapitata in the presence of AgPEG 100µgL-1. Mean values ± SD (N = 3). HS1 = 1 mgL-1 HS; HS2 = 10 mg L-1 HS. * Statistically significant difference compared to control (p <0.05).

AgCit EC_{50} values under *R. subcapitata* microalgae after 96 h were equivalent to 32.40 µg L^{-1}. Significant differences in growth rate of the control sample exposed to 100 µg L^{-1} PEG in the presence and absence of humic substances can be observed. Gunsolus et al. (2015) conducted studies in which they demonstrated that the stability of silver nanoparticles promoted by humic substances depends on the type of substance and the coating agent of the nanoparticles. Silver nanoparticles were exposed to humic and fulvic acids and the aggregation rate of the nanoparticles depended on the chemical composition of these substances. In studies conducted by Sharma et al. (2014), it was observed that the interaction between silver nanoparticles with humic and fulvic acids caused changes in the charge and stabilization of these nanoparticles, the proton release of these sodium citrate coated nanoparticles decreased when they were placed in acid-containing solutions. (Sharma *et al.*, 2014).

The interaction between humic substances and the silver nanoparticle coatings plays a large role within these results, since it is this nanoparticle coating that directly interacts with the natural organic matter present in the solution. Studies have shown that the use of humic substances as reducing and stabilizing agents during the preparation of nanoparticles is attractive because of their ability to improve colloidal stability of the solution, as well as reducing the use of toxic reagents during this procedure (Da Costa Cunha *et al.*, 2014).

According to Lapresta-Fernandez et al.(2012), the antimicrobial and fungicidal function of these AgNPs would be due to the release of these Ag^{+} ions into the medium.

Other possible environmental impacts of these nanoparticles in the medium is the large aggregation capacity of the particles, which influences the sedimentation rate and the mobility of these nanomaterials in the environment (Baalousha *et al.*, 2008; Maurer-Jones *et al.*, 2013). Interactions between nanoparticles and humic substances can prevent problems from extending to this extent and help to counteract the adverse effects of silver nanoparticles on the medium.

4.3.4 Bioaccumulation

For the bioaccumulation results (Figure 4. 11), when the *R. subcapitata* was exposed to silver nitrate in the presence and absence of organic matter, the bioaccumulation is even higher in the presence of organic matter. However, when *R. subcapitata* was exposed to AgCit, the bioaccumulation decreases with the increase of humic material. In both cases, the accumulation increases with time. In terms of mass, the Ag accumulation is much higher for ionic Ag ($AgNO_3$) exposition than silver nanoparticles.

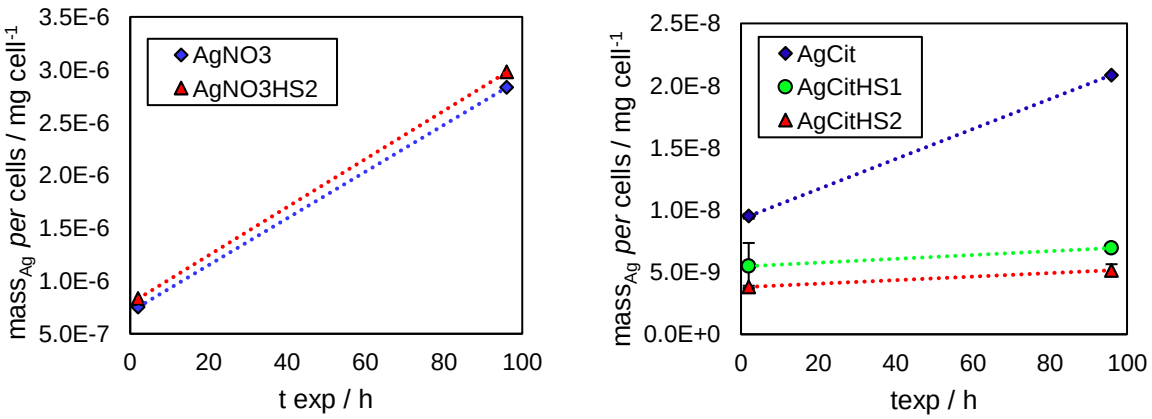


Figure 4. 11 - Bioaccumulation results of a) AgNO3 and b) AgCit in the presence and absence of aquatic humic substances.

Hiriart-Baer et al. (2006) have observed that *R. subcapitata* was able to accumulate more Ag when the concentration of Ag thiosulfate complexes in the medium was increased. This is due to the fact that the pore diameter of the algae cell wall is 5 to 20 nm, so only smaller nanoparticles can be internalized by the cells (Navarro *et al.*, 2008). Because they are free, silver ions may have caused toxicity

even in the presence of humic substances. Nanoparticle agglomeration should also be considered when analyzing bioaccumulation toxicity, as they are able to adsorb on the outer surface of the algae (Handy *et al.*, 2012)

Thus, the aggregation rate may impact cell division with consequent decrease in growth rate and photosynthesis (Aruoja *et al.*, 2009). Chen et al. (2013) have studied the toxicity of silver ions in the presence of humic substances and have found similar results. The authors have shown that the increase in silver uptake observed in the presence of humic substances did not decreased the algal concentration. Furthermore, the results suggested that the increase in silver uptake observed in the presence of organic matter is due to the surface-bound, it means, not truly internalized by the algae. During the procedures for bioaccumulation experiments in this work, EDTA solution was used as a complexing agent to remove Ag attached / adsorbed on the algae cells walls.

Considering toxic response of *Raphidocelis subcapitata* and comparing the bioaccumulation of nanosilver capped with citrate and ion silver it is reasonable to think that the silver amount accumulated is much higher for ion and that it was induced high toxicity comparing to citrate response. The uptake of silver nanoparticle ($1.0x10^{-8}$ mg of Ag) was much lower than $AgNO_3$ ($1.0x10^{-2}$ mg of Ag): twice of value. For AgPeg, there is an effect not clearly evidenced, since no bioaccumulation was observed (bioaccumulation measurements lower than quantification limit). Some author reveals toxicity governed by physical or mechanical effects, having the nanoparticle avoiding the physiological patterns due to the barrier formed in the algal surface.

The dissolution results obtained in the Chapter 3 (Figure 3. 3) shows a dissolution of AgPEG and the consequent bioavailability of silver ions on the solution. Almost 40 % of dissolution occurs, releasing enough silver ion to induce toxic effect (EC_{50} for Ag^+ =17.83 μgL^{-1}). Another explanation was raised by Campbell et al., (1997) and Knauer and Buffle (2001), which have observed that organic matter can influence the bioavailability of organisms through complexation and, in the case of algae, can adsorb on the body surface, blocking the surface pathways through which uptake can occur. The same mechanism, could occur for algae, where the nanomaterial could interact with the algae surface and avoid essential activity or essential element internalization.

4.4 Conclusion

Firstly, the toxic effect for silver ions observed did not changed with the presence of natural organic matter. The difference of capping agents as well as nanoparticle concentration shows different toxicological responses by observing a AgPeg toxicity compared to the same Ag concentration with Citrate which did not presented toxic effect. The toxicity of PEG can be related to the dissolution behavior assessed in previous chapter, also associated to the solution exposed and exposition time. For bioaccumulation, the presence of natural organic matter enhanced the silver amount in the algae probably due to surface bound of HS-Ag. Finally, the presence of NOM influences in the silver bioaccumulation, and for citrate capped nanoparticles, there is a decrease of silver accumulated with the increase of humic material.

CHAPTER 5: WATER BORNE AND DIET BORNE OF SILVER NANOPARTICLES IN THE PRESENCE OF NATURAL ORGANIC MATTER EXPOSURE OF DAPHNIA SIMILIS

Abstract

Since NPs may reach the aquatic environment, potential impact e.g. aquatic environmental effects should be considered. Natural organic matter is ubiquitous in aquatic systems. The diversity of coatings as well as nanoparticle concentration show different toxicological responses by observing a higher PEG nanoparticle toxicity compared to the same Ag concentration with Citrate coatings. A greater sensitivity of micro crustaceans to algae can also be observed. The toxic effects were evaluated by mortality (EC50) and reproduction, the last one through the offspring (number of neonates) generated during the tests. For algae-microcrustacean a simplified food chain experiment, the algae were exposed to a Ag+ and/or AgNP concentration in order to have a enough accumulation. It will be done with a control without Ag, AgNO3, AgCit and AgPEG using one (high amount) of Ag and the 4 NOM exposition types: HS1, HS2, EPS and HS+EPS. the presence of organic matter represented by humic substances, silver ions dispersed on solution induced high toxicity and sensitivity for the target organism. In agreement with literature related, nanoparticle dissolution can be related to the toxic effect observed in both capped nanosilver tested. The importance of chronic study is also demonstrated due to the toxic effect on Daphnia reproduction was observed in allowed concentrations. In this way, humic substances can minimize the toxicity for acute response but, in some cases, enhance the toxic effect in adult as we could see small concentrations of AgPeg showing toxicity only in the presence of humic substances.

Keywords: food chain; organic matter; nanomaterial; ecotoxicology.

5.1 Introduction

Since large amounts of NPs may reach the environment, the environmental potential impact e.g. aquatic environmental effects should be considered. The environmental fate, state of agglomeration or aggregation, and dissolution in

environmental and biological media are dependent on how nanosilver is prepared, what types of surface coating are used, and the conditions under which they are used. As a result, environmental fate is highly variable within a range of surface functionalization that can make the same material biocompatible or biohazardous. Dissolution has been reported as the main mechanism that causes toxicity of nanoparticles (Ratte, 1999).

A brief research from Web of Science presented in the Figure 5. 1, shows in the last 10 years the number of publications related to the term NANPARTICLES have considering enhanced, showing the great interest on this topic. The same research performed, that time using the terms NANOPARTICLES and SILVER give a mean 11 % of the papers publishing in the same proporstion of studie of NANOPARTICLES. However, the studies related to ECOTOXICITY or ECOTOXICOLOGY varieted (since 2010-2019) from 5 to 50 works, shows that those studies are still scarce.

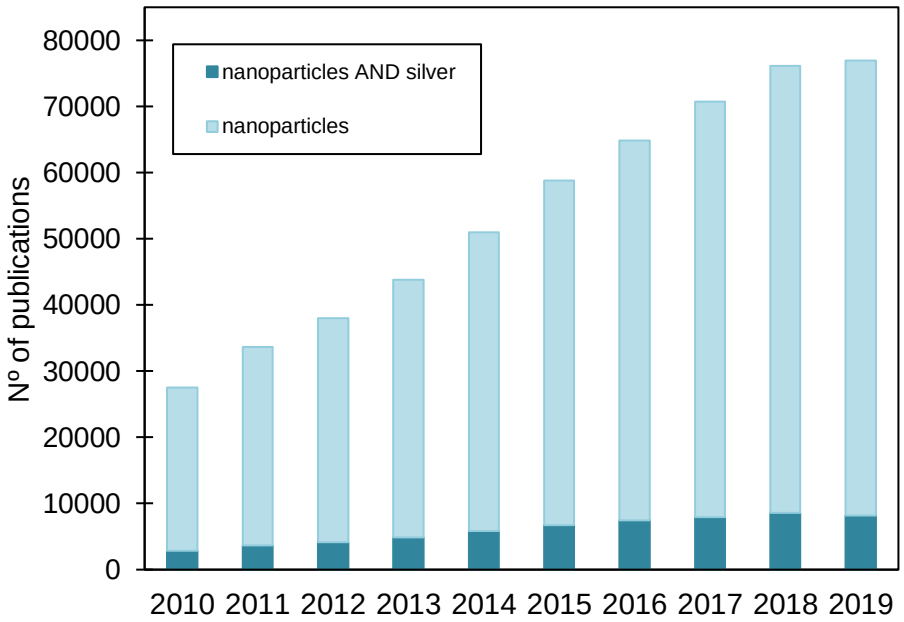


Figure 5. 1 - Number of publications *versus* year of publication related to “Nanoparticles” and “Nanoparticles + Silver” studies. Source: Web of Science (2020).

Studies have performed toxic impact of NPs on a variety of aquatic organisms including decomposers (bacteria), primary producers (algae) and secondary consumers (micro-invertebrates and fish. *Daphnia sp*. is a microcrustacean (order Cladocera) zooplankton freshwater. Micro-crustaceans play an important role in the

food chain, they fed on algae (producers) and they are used as food for other consumers, such as fish and other vertebrates. Trophic transfer studies allow us to differentiate the importance of different exposure routes which is useful in risk analysis (Berthet, 2015; Kalman *et al.*, 2015). Possible risk associated with nanomaterial exposure may arise since during nanomaterial fabrication, handling of nanomaterials until the subsequent processing to create derivate products, product usage and as the result of post usage or waste disposal practices (Wiesner and Bottero, 2007).

Humic substances compose a class a natural organic matter that are known for having important roles in alleviating metal toxicity to aquatic organisms. Recent literature reports suggest that humic substances may also exert direct influences on biota (Angel *et al.*, 2013). The NOM influencing the toxicity of nanoparticles, becomes essential to understand the types of NPs transformations involved and to stablish associations with the toxicity effects. By understanding these transformations, it is possible to establish more clearly mechanisms that help to better understand emerging contaminants in response to ecotoxicological responses. Thus, the aims of the present work were to evaluate the toxicity of Ag^{+}, AgCit and AgPeg nanoparticles in the presence and absence of organic matter and verify the influence of waterborne and dietborne exposition using a simple food chain Algae-Daphnia for both juvenil and adult Daphnia similis test-organisms. These data provide a reference for the further application of risk evaluations of nanomaterials in fresh water ecosystem by considering the organic matter found in aquatic environment.

5.2 Materials and Methods

5.2.1 Daphnia similis culture and maintenance

Daphnia similis cultures were purchased from the Ecotoxicology Laboratory of the IPEN Center for Chemistry and Environment. *D. similis* cultures were performed applying methodological procedures established in OECD 202 (2004) guidelines, which specifies acute toxicity test for Daphnia sp. as well as protocol for reproduction test contained in OECD No. 211 (2012). In addition, the Brazilian protocol ABNT (2016) standard that presents protocol and conditions for acute toxicity testing for *Daphnia spp* was also considered, due to specific cultive information contents for the *Daphnia*

similis specie. Thus, massive cultures of these individuals were maintained using maintenance procedures of cultivation water renewal, age control to perform definitive assays, daily feeding as well as sensitivity tests to ensure reproducibility of the assays. Table 5. 1 presents the general description of the conditions used in the cultivation of *Daphnia similis*. Controlled cultures were carried out in 600 mL beckers containing 500 mL of reconstituted soft water (RSW) and 30 parthenogenetic females at each container. The organisms were fed three times per week on the green microalgae *Raphidocelis subcapitata* dosing $5x10^6$ cells per organism and once per week with filtered fish food dosing 0.02 mL per organism. Total RSW was replaced weekly when new brood was separate. The cultures were maintained at 21±1 ºC and photoperiod 16:8 (light: darkness) of diffuse light.

Table 5. 1 - Cultivation condition for D. similis based on ABNT (2016) protocol.

Parameter	**Cultive condition**
pH	7.0-7.6
Hardness of reconstituted water*	40 a 48 mg $CaCO_3$ L^{-1}
Temperature	de 21 °C
Light	16 h of light and 8 h darkness
Food	Algae *Raphidocelis subcapitata*: 1 a $5x10^6$ cells/organism daily applied. Fish food** – 0,02 mL per organism every water renewal.
Number of individuals per bequer	20-30
Water renewal per week	2
Organisms discard	after 28th day

(*) Hardness adjusted using calcium sulfate dihydrate, sodium bicarbonate, magnesium sulfate heptahydrate and potassium nitrate.
(**) Digested fish food solution (1 hour of agitation + 1 hour of decantation).

5.2.2 Culture feasibility study

During the months of implementation of the culture, the parameters of mortality and reproduction of the organisms were monitored, providing greater reliability in the assays to be obtained. To this end, feasibility tests were performed during the first 3 months of culture implementation. The assay consisted of the separation of 10-20 organisms aged 6-24 h (neonate) distributed into individual containers and monitored over a week. The organisms were exposed to different culture media by water renewal as well as mortality and counting of the number of newborns produced per organism

every two days. More details about acclimatation procedure is available on Appendix 5.

5.2.3 Sensitivity test conditions

Sensitivity control of the test organism, using concentration ranges of a reference toxicant, provides greater precision and reliability in the test results obtained over time (Zagatto and Bertoletti, 2006). Thus, sensitivity tests were performed for the organism Daphnia similis using NaCl as reference toxicant throughout ecotoxicological studies carried out. Figure 5. 2 presents a schematic summary of the sensitivity test, as well as the main conditions for its performance. Daphnia similis neonates (6-24 h-old) were exposed to the following NaCl concentrations (g L^{-1}): 0 (control), 0.25, 0.5, 1.0, 2.0, and 4.0, with four replicates per concentration, and five neonates per replicate. Test vials were kept at 20±1 ºC, under 16 h/8 h (light/dark) photoperiod for 48 h. Neonates were not fed during the test. After 48 h, immobility of individuals was determined and the EC_{50} estimated by the Trimmed-Spearman Karber method (Hamilton *et al.*, 1977).

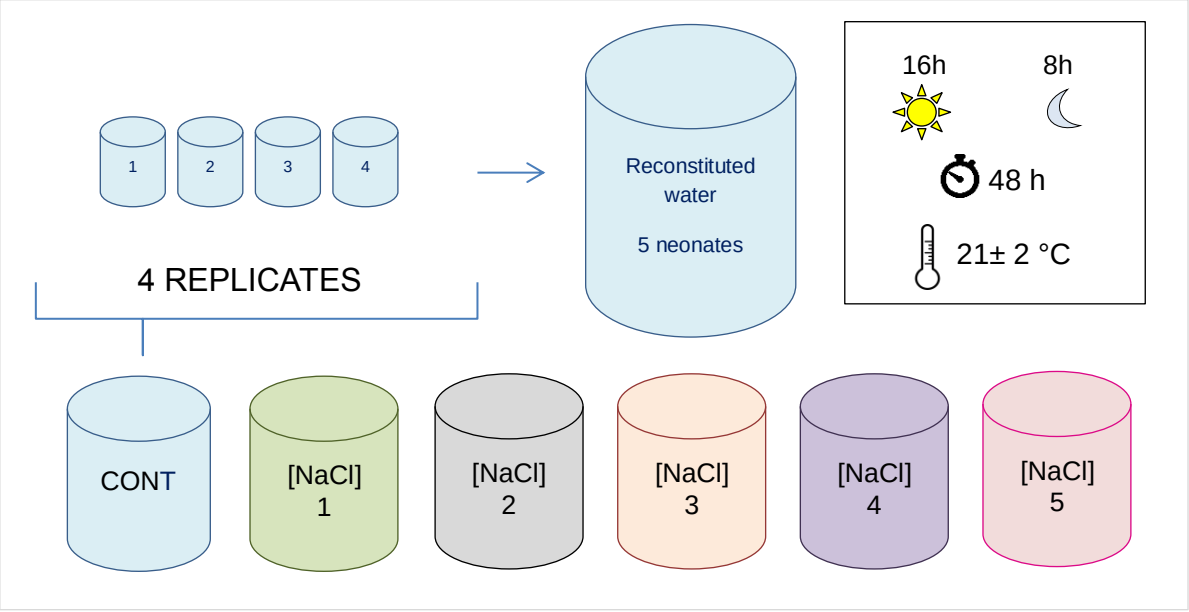


Figure 5. 2- General scheme of Sensitivity Test for D. similis. Five concentrations (NaCl) plus one Control under temperature, photoluminescence and experiment time conditions.

5.2.4 Acute toxicity bioassays

Acute toxicity bioassays were performed according to the Protocol 202 of the Organization for the Economic Cooperation and Development (Oecd, 2004) plus specific conditions for *D. similis* cultivation contained in Brazilian Standard protocol NBR 12713 (Abnt, 2016) was used as a complement. Several concentrations of $AgNO_3$ were also performed in order to determine the EC_{50}, in terms of silver ions.

The test assesses the immobility effect of the organism exposed to concentrations of a given substance and can be expressed by EC_{50} (for Daphnia) using a series of EC_{50} data obtained. From an $AgNO_3$ stock solution prepared as 4.2.3, 7 concentrations were prepared in a range of 1 to 100 μgL^{-1} in Ag^{+}. For each concentration, 10 organisms aged between 6 and 24 h were individually distributed in vials containing 20 mL of test solution. The orgnanisms were kept in an incubator under the same conditions as described in Table 5. 1 for a period of 48 h without food and water renewal. Figure 5. 3shows a schematic summary of the $AgNO_3$ assay as well as the main conditions for its performance. To determine the EC_{50} of toxicity assays, the Trimmed Spearman-Karber method (Hamilton, 1977) was used.

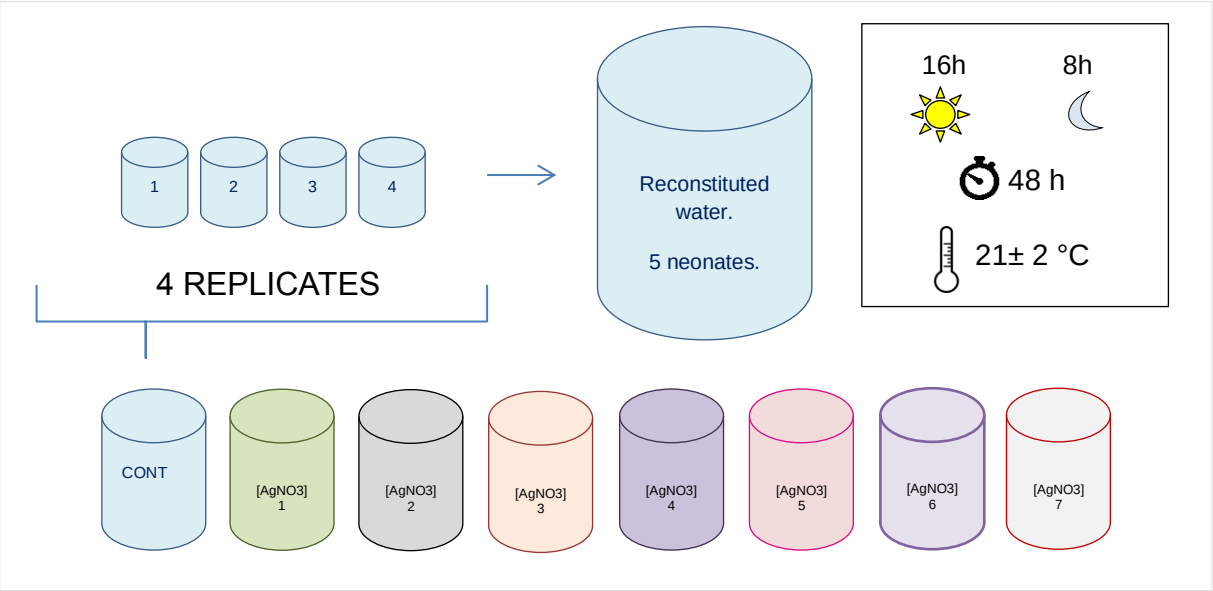


Figure 5. 3 - Definitive test layout for D. similis. Seven concentrations (AgNO3) plus one Control under temperature, photoluminescence and experiment time conditions.

5.2.5 Long-term exposure bioassays

Long-term exposure bioassays were performed according the Protocol 211 of the Organization for the Economic Cooperation and Development (Oecd, 2012) as reference *Daphnia magna* reproduction protocols. In that way, *D. similis* were fed 0.02 mL of a yeast per organism, Tetramin, digested trout chow slurry two out of every three days, with water renewal every two or three days. Reproduction and survival were monitored during 21 days.

5.2.6 Waterborne and dietborne comparative assays

The Figure 5. 4 ilustrate a resume of Waterborne and Dietborne algae-microcrustacean bioassays. Firtly, for Dietborne, the algae were exposed to Ag^{+} and/or AgNP concentrations during 96 h. Each solution was centrifudged, the algae was separated and the cells concentration was determined. For Waterborne (W), neonates (<24 h) and adults (day 7) were exposed during 48 h at two concentrations (1 and 100 μgL^{-1}) of $AgNO_3$, AgCit and AgPeg nanoparticles in both presence and absence of humic substances. For Dietborne (D), algal solution (in order to reach $1x10^7$ cells) was added in the test solution to the same 24 h (neonate) and day 7 (adult) organisms. Exposition control solution without Ag, AgNO3, AgCit and AgPEG using humic substances concentration: 1 mgL^{-1} (HS1) and 10 mgL^{-1} (HS2) was also performed.

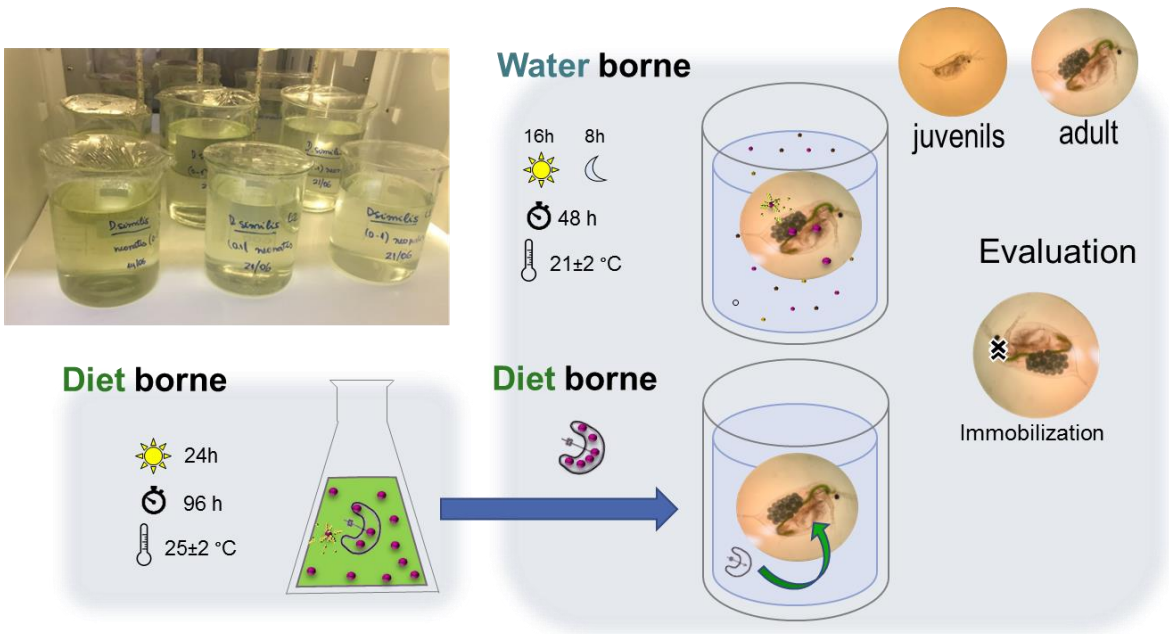


Figure 5. 4 - Schedule for Waterborne and Dietborne Daphnia similis assays.

5.2.7 Phsysicochemical parameters

Standard physicochemical parameters were monitored in each treatment level at the end of the test. The pH, dissolved oxygen, conductivity and hardness were measured.

5.2.8 Statistical analyses

For acute response bioassay using *Daphnia similis* 48 h test, the EC_{50} response for Ag^{+}, AgCit and AgPeg were calculated using Trimmed Spearman Karber (TSK) software (Hamilton *et al.*, 1977).

For long-term exposure. the toxic response was firstly using Fisher's Exact Test performed by Toxstat 3.5 (West and Gulley, 1996) in order to identify eventual acute toxic effect. The treatments that have shown differences ($p<0.05$) were not selected to proceed with reproduction response effect. The reproduction data (offspring) of the remaining treatment was tested according to normality (Chi-square and Shapiro Wilk's test) and homogeneity (Hartley's test and Bartlett's test). Then, the data were submitted to one-way analysis of variance (ANOVA Bonferroni t-Test) for comparison between control means in relation to treatments, with a significance level of 5% ($p <0.05$) in all analyzes. Toxstat 3.5 was also used to compare the immobility assessment of control and test exposition in water and dietborne exposition using Fisher's Exact Test significant level ($p<0.05$).

5.3 Results and Discussion

5.3.1 Sensitivity test using reference toxicant

The data of sensitivity test expressed in EC_{50} obtained using NaCl as reference toxicant is available inFigure 5. 5. The value is close to the reference values for the same substance from the original laboratory, attesting to its health and good acclimatization.

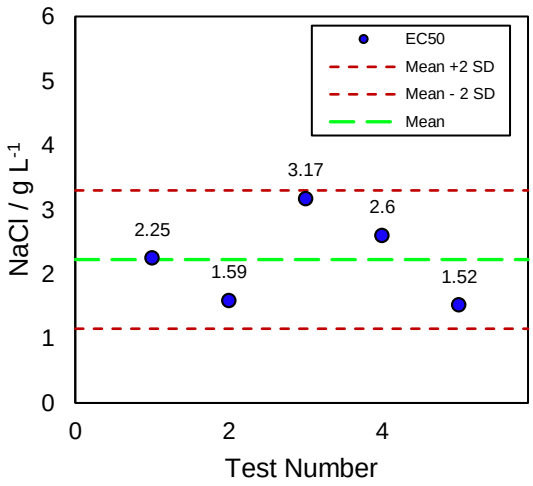


Figure 5. 5 -Control chart of EC50 Daphnia similis using NaCl as toxicant reference substance.

5.3.2 Acute bioassays

The EC_{50} graph for *D. similis* can be seen in Figure 5. 6. For $AgNO_3$ the value of EC_{50} = 6.52 µg L^{-1} shows a higher sensitivity of microcrustacean compared to the microalgae EC50 results studied in chapter 4. Studies of Zhao and Wang (2011)have showed that daphnids were sensitive to $AgNO_3$ exposure obtaining a 48-h LC_{50} of 2.51 µgL^{-1}.

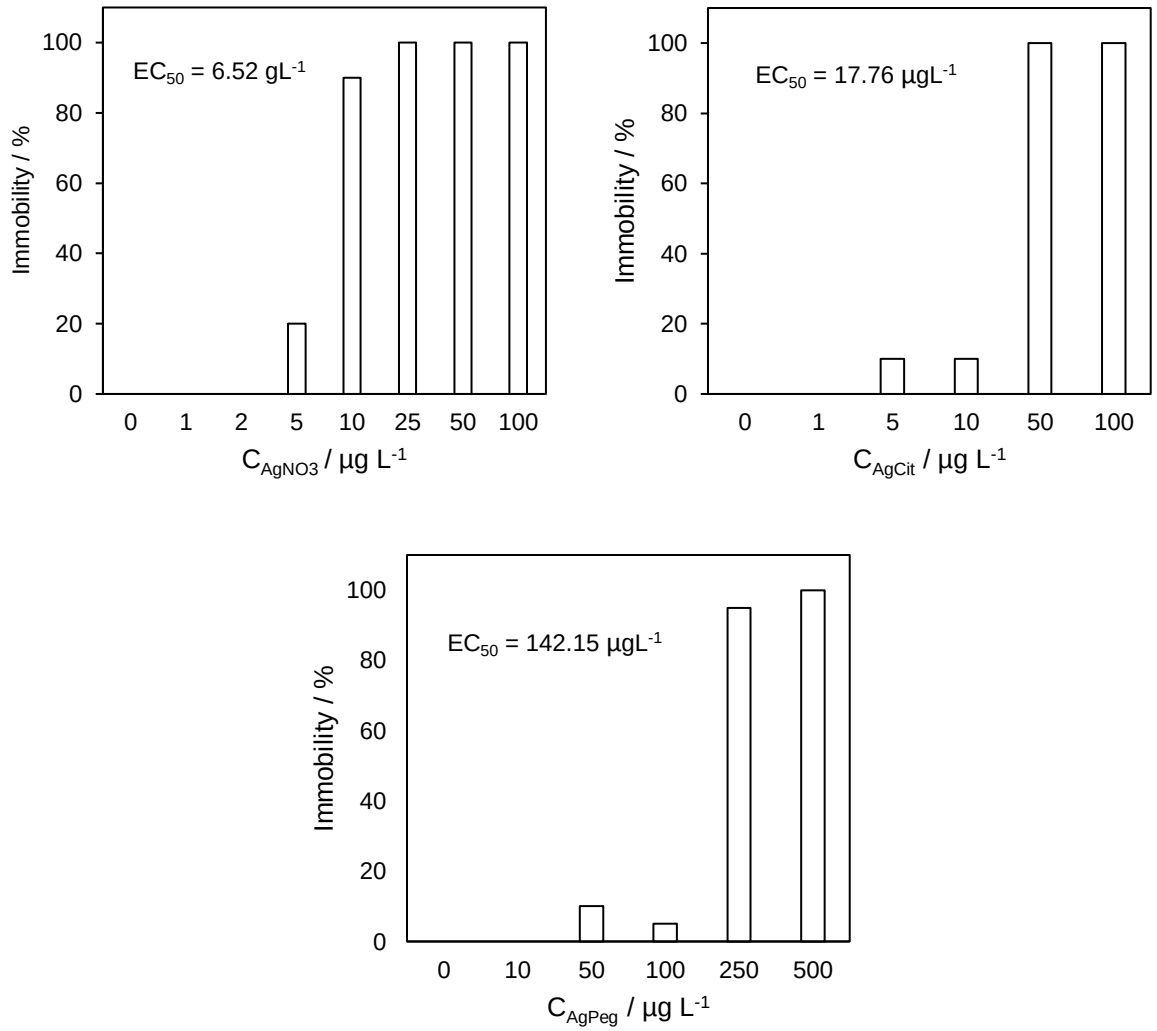


Figure 5. 6 -– EC50 to D. similis as a function of the mean Ag+ and as a function of the nanosilver (B) AgCit (c) AgPEG).

As mentioned before, due to the fact that Daphnias are filtering organisms, internalization flows may be higher, leading to a short-term exposure and toxicity effect. Gao et al. (2009) have found that in the presence of high concentrations of natural organic matter (NOM) reduces the toxicity of AgNPs to *Ceriodaphnia dubia* (microcrustacean), but at 50 mg L^{-1} AgNPs concentrations at pH 7 there is an increased toxicity to Daphnia-like organisms even in larger amounts of NOM. The toxicity studies of AgNPs in the trophic chain combined with the dissolution rates of AgNPs already studied may help to understand the mechanisms of internalization and toxicity effects of these microcrustaceans against nanomaterials.

5.3.3 Long-term exposure bioassay

The long-term exposure results are presented in Appendix 6. Table 5. 2 and Table 5. 3 shows the survival and reproduction data for 1 and 100 μgL^{-1} AgNPs, respectively. According to the data, it's possible to observe for the highest concentration, significant differences compared to the control in all treatments for acute test performed by Fisher Extact's Test. For AgCit no toxicity is observed for low concentration of AgNP. As it is possible to observe on thhe Table 5. 3, high concentration of AgCit induces high toxicity to *D. similis*. In some cases, it was possible to observe that there are still some reproduction, but high mortality is observed at the end of the test. The tests using AgPEG shows no toxicity for low concentrations. No toxicity for the highest concentration in the presence of HS2 is observed.

Table 5. 2 - Survival and reproduction data for 1 µgL-1 AgNPs.

Concentration	Survival / %	Offspring / neonate female^{-1}
Control	97	79
AgCit1	80	99
AgCit1HS1	87	94
AgCit1HS2	97	80
AgPeg1	97	78
AgPeg1HS1	97	90
AgPeg1HS2	83	84

Table 5. 3 - Survival and reproduction data for 100 µgL-1 AgNPs.

Concentration	Survival / %	Offspring / neonate female^{-1}
Control	95	48
AgCit100	0	0
AgCit100HS1	0	3
AgCit100HS2	15	5
AgPeg100	50	31
AgPeg100HS1	30	33
AgPeg100HS2	70	45

5.3.4 *Waterborne and diet exposition comparison test*

The immobilization of Daphnia similis was recorded after 48 h of exposure for $AgNO_3$, AgCit and AgPeg exposition. According to the immobilization results organized in the Figure 5. 7, neonates (Figure 5. 7a, b and c) are more sensitive than adults (Figure 5. 7d, e and f), except when citrate nanosilver is directly dispersed in water (Figure 5. 7b and e). In that case, when the exposition of the highest concentration occurred there is no toxic effect in the presence of HS2 for neonates exposition but toxic effect where adults were exposed, in all treatment performed with humic substances.

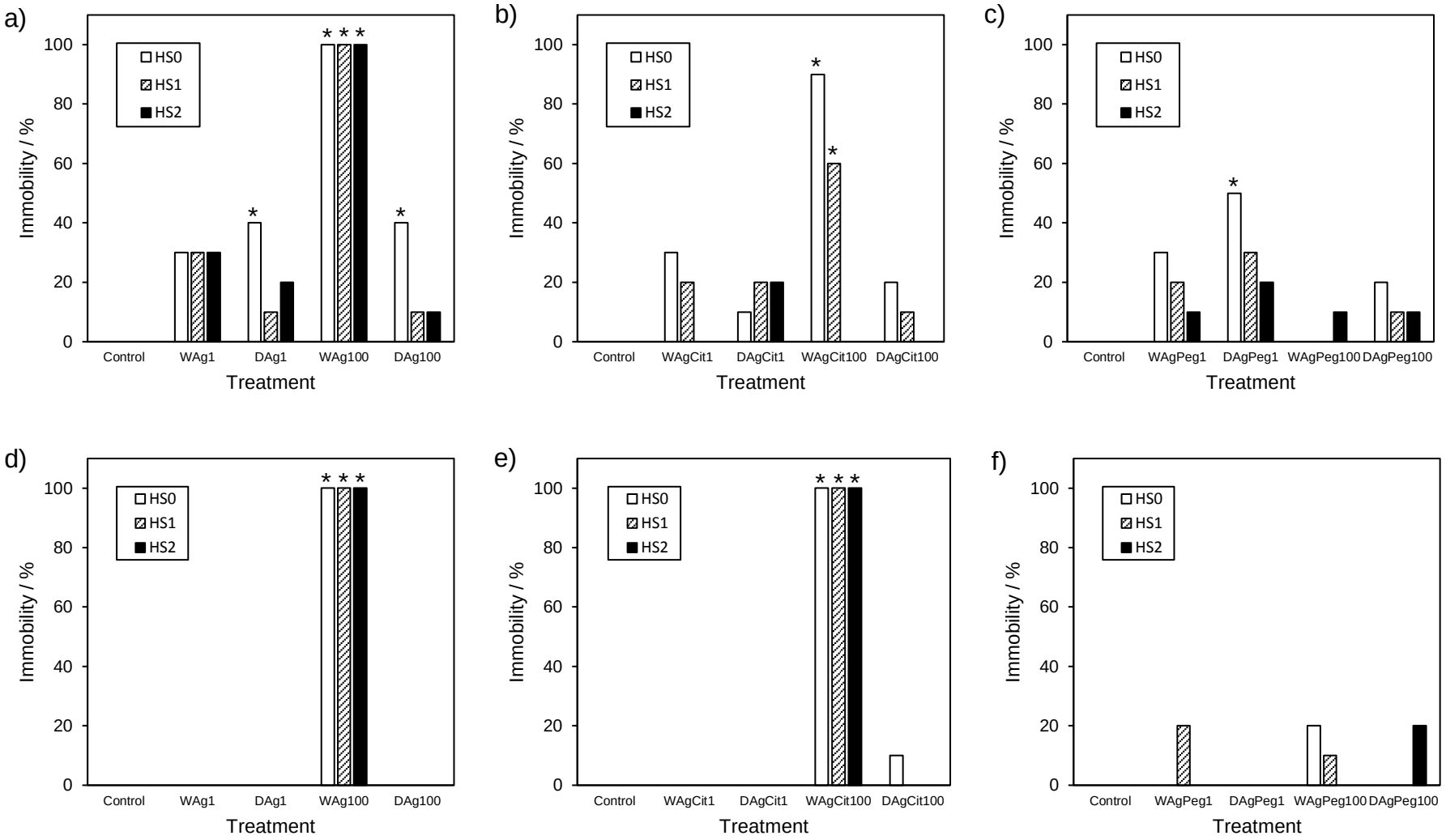


Figure 5. 7 - Waterborne and dietborne D. similis exposition onto Ag+, AgCit and AgPeg in absence (HS0) and presence of 1 and 10 mgL-1 (HS1 and HS2, respectively) of Humic substances.

The immobilization results show that neonates (Figure 5. 7a, b and c) are more sensitive than adults (Figure 5. 7d, e and f) taking to account age exposition. According to Huang et al (2016), organisms exposed during the early developments stage have been reported to be more intolerant to damage effects of pollutants when compared to adult phase. Zhao and Wang (2012) have also performed toxicity exposition using different life-stages. The acute toxicity bioassays with different life stages showed a pattern of increasing the LC_{50} values as function of organisms' age. In other words, neonates were more sensitive than mature life stages (in these work, 7-8 days age) as it was referred by the correspondent statistical analyses, which showed significant differences among the groups tested. Ribeiro et al. (2017) have reported that Ag ion induced higher toxicity than AgNP when they exposed D. magna in Waterborne, i.e. when the AgNPs is dispersed in water.

5.4 Conclusion

Based on the results obtained for *D. similis* assays, the presence of organic matter represented by humic substances, silver ions dispersed on solution induced high toxicity and sensitivity for the target organism. In agreement with literature related, nanoparticle dissolution can be related to the toxic effect observed in both capped nanosilver tested. The importance of chronic study is also demonstrated due to the toxic effect on Daphnia reproduction was observed in allowed concentrations. In this way, humic substances can minimize the toxicity for acute response but, in some cases, enhance the toxic effect in adult as we could see small concentrations of AgPeg showing toxicity only in the presence of humic substances.

CHAPTER 6: FINAL REMARKS

The present work allows us to confirm how the natural organic matter studied influences the transformation of nanomaterials in terms of charge density and dissolution and how the characterization of these nanomaterials are important under environmental conditions. Furthermore, dissolution may be influenced by the aquatic ecosystem chemistry: pH and/or ionic strength. Even though, the two different organic matter assessed (EPS and HS) presented the same behavior as complexing agent of nanoparticles and contributed to their stabilization independently of the media composition.

The toxicity assessment using the microalgae *R. subcapitata* showed that the toxic effect observed for silver ions did not change with the presence of natural organic matter. The difference of capping agents as well as nanoparticle concentrations showed different toxicological responses by observing a AgPeg toxicity compared to the same Ag concentration with Citrate which did not presented toxic effect. The toxicity of AgPeg can be related to the dissolution behavior assessed previously, also associated to the solution composition and exposition time. For bioaccumulation, the presence of natural organic matter enhanced the silver amount in the algae probably due to surface bound of HS-Ag. Finally, the presence of NOM influences in the silver bioaccumulation, and for citrate capped nanoparticles, there is a decrease of silver accumulated with the increase of humic material.

Based on the results obtained for *D. similis* assays, the presence of organic matter, silver ions dispersed on solution induced high toxicity and sensitivity for the target organism. In agreement with literature related, nanoparticle dissolution can be related to the toxic effect observed in both capped nanosilver tested. The importance of chronic studies is also demonstrated since a toxic effect on Daphnia reproduction was observed under our experimental condition. In this way, humic substances can minimize the toxicity for acute response but, in some cases, enhance the toxic effect in adult as we could see small concentrations of AgPeg showing toxicity only in the presence of humic substances.

The simplified food chain studies show tahta there are evidences of biomagnification of silver based exposition dietary. This exposition may cause toxicity when the organism is exposed in a young phase but also when in adult, the

nanoparticle toxicity can be enhanced even in the presence of natural organic matter acting as a stabilizer avoiding then dissolution.

Looking forward to future perspectives, the investigation could evaluate the fate of silver NPs in terms of sedimentation rate, aggregation (homo/ hetero). In addition, experiments involving the characterization of NPs behavior in the Media exposition from another organisms (seawater, groundwater) are capable to improve the knowledge of the nanoparticles transformation.

For ecotoxicology assays, complementary studies involving bioaccumulation and modelisation (surface binding) for algae and cryomicroscopy, depuration, excretion rate as well modelisation to daphnia may also contribute to the knowledge and development of environmental safety of nanomaterials monitoring.

By understanding these transformations, it is possible to establish more clearly mechanisms to better understand emerging contaminants behavior in the environment.

REFERENCES

ABBT-BRAUN, G.; FRIMMEL, F. H.; SCHULTEN, H.-R. Structural investigations of aquatic humic substances by pyrolysis-field ionization mass spectrometry and pyrolysis-gas chromatography/mass spectrometry. **Water Research,** v. 23, n. 12, p. 1579-1591, 1989/12/01/ 1989. ISSN 0043-1354. Available at: < http://www.sciencedirect.com/science/article/pii/0043135489901243 >.

ABBT-BRAUN, G.; LANKES, U.; FRIMMEL, F. H. Structural characterization of aquatic humic substances – The need for a multiple method approach. **Aquatic Sciences,** v. 66, n. 2, p. 151-170, 2004/06/01 2004. ISSN 1420-9055. Available at: < https://doi.org/10.1007/s00027-004-0711-z >.

ABNT. **Aquatic ecotoxicology - Acute toxicity - Test with Daphnia spp (Cladocera, Crustacea):** 27 p. 2016.

_____. **Aquatic ecotoxicology - Chronic toxicity - Test with algae (Chlorophyceae)**. 12713**:** 27 p. 2018.

ADAMS, F. C.; BARBANTE, C. Nanoscience, nanotechnology and spectrometry. **Spectrochimica Acta Part B: Atomic Spectroscopy,** v. 86, p. 3-13, 2013/08/01/ 2013. ISSN 0584-8547. Available at: < http://www.sciencedirect.com/science/article/pii/S0584854713001031 >.

ANGEL, B. M. et al. The impact of size on the fate and toxicity of nanoparticulate silver in aquatic systems. **Chemosphere,** v. 93, n. 2, p. 359-365, 2013/09/01/ 2013. ISSN 0045-6535. Available at: < http://www.sciencedirect.com/science/article/pii/S0045653513007212 >.

ARUOJA, V. et al. Toxicity of nanoparticles of CuO, ZnO and TiO2 to microalgae Pseudokirchneriella subcapitata. **Science of The Total Environment,** v. 407, n. 4, p. 1461-1468, 2009/02/01/ 2009. ISSN 0048-9697. Available at: < http://www.sciencedirect.com/science/article/pii/S0048969708010784 >.

ASHARANI, P. V. et al. Toxicity of silver nanoparticles in zebrafish models. **Nanotechnology,** v. 19, n. 25, p. 255102, Jun 2008. ISSN 0957-4484. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/21828644 >.

ASSUMPÇÃO, R. M. V.; MORITA, T. **Manual de soluções, reagentes e solventes: padronização-preparação-purificação**. Edgard Blücher, Ed. da Universidade, 1968. Available at: < https://books.google.com.br/books?id=crdcAAAAMAAJ >.

BAALOUSHA, M. et al. Aggregation and surface properties of iron oxide nanoparticles: influence of pH and natural organic matter. **Environ Toxicol Chem,** v. 27, n. 9, p. 1875-82, Sep 2008. ISSN 0730-7268. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/19086206 >.

_____. Effect of monovalent and divalent cations, anions and fulvic acid on aggregation of citrate-coated silver nanoparticles. **Science of The Total Environment,** v. 454-455, p. 119-131, 2013/06/01/ 2013. ISSN 0048-9697. Available at: < http://www.sciencedirect.com/science/article/pii/S0048969713002799 >.

BADAWY, A. M. E. et al. Impact of Environmental Conditions (pH, Ionic Strength, and Electrolyte Type) on the Surface Charge and Aggregation of Silver Nanoparticles Suspensions. **Environmental Science & Technology,** v. 44, n. 4, p. 1260-1266, 2010/02/15 2010. ISSN 0013-936X. Available at: < https://doi.org/10.1021/es902240k >.

BENEDETTI, M. F. et al. Metal ion binding by natural organic matter: From the model to the field. **Geochimica et Cosmochimica Acta,** v. 60, n. 14, p. 2503-2513, 1996/07/01/ 1996. ISSN 0016-7037. Available at: < http://www.sciencedirect.com/science/article/pii/0016703796001135 >.

BERTHET, B. Chapter 9 - Reference Species. In: AMIARD-TRIQUET, C.;AMIARD, J.-C.*, et al* (Ed.). **Aquatic Ecotoxicology**: Academic Press, 2015. p.205-227. ISBN 978-0-12-800949-9.

BEZERRA, P. S. S.; TAKIYAMA, L. R.; BEZERRA, C. W. B. Complexacao de ions de metais por materia organica dissolvida: modelagem e aplicacao em sistemas reais. **Acta Amazonica,** v. 39, p. 639 - 648, 2009. ISSN 0044-5967. Available at: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0044-59672009000300019&nrm=iso >.

BOULDIN, J. L. et al. Aqueous toxicity and food chain transfer of Quantum DOTs in freshwater algae and Ceriodaphnia dubia. **Environ Toxicol Chem,** v. 27, n. 9, p. 1958-63, Sep 2008. ISSN 0730-7268. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/19086211 >.

BURBA, P.; SHKINEV, V.; SPIVAKOV, B. Y. On-line fractionation and characterization of aquatic humic substances by means of sequential-stage ultrafiltration. **Fresenius' Journal of Analytical Chemistry,** v. 352, n. 3, p. 402-402, 1995/01/01 1995. ISSN 1432-1130. Available at: < https://doi.org/10.1007/BF00322245 >.

CAMPBELL, P. G. C.; TWISS, M. R.; WILKINSON, K. J. Accumulation of natural organic matter on the surfaces of living cells: implications for the interaction of toxic solutes with aquatic biota. **Canadian Journal of Fisheries and Aquatic Sciences,** v. 54, n. 11, p. 2543-2554, 1997/11/01 1997. ISSN 0706-652X. Available at: < https://doi.org/10.1139/f97-161 >. Accessed on: 2020/03/21.

CHALOUPKA, K.; MALAM, Y.; SEIFALIAN, A. M. Nanosilver as a new generation of nanoproduct in biomedical applications. **Trends in Biotechnology,** v. 28, n. 11, p. 580-588, 2010/11/01/ 2010. ISSN 0167-7799. Available at: < http://www.sciencedirect.com/science/article/pii/S0167779910001228 >.

CHEN, Z. et al. Influence of Humic Acid on Algal Uptake and Toxicity of Ionic Silver. **Environmental Science & Technology,** v. 47, n. 15, p. 8835-8842, 2013/08/06 2013. ISSN 0013-936X. Available at: < https://doi.org/10.1021/es401085n >.

COMISSION, E. Definition - Nanomaterial. **2011/696/EU** 2011. Avaialble at: < https://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm >.

CONAMA. **RESOLUÇÃO No. 357**. AMBIENTE, M. D. M.**:** 27 p. 2005.

_____. **RESOLUÇÃO No. 430**. AMBIENTE, M. D. M.**:** 9 p. 2011.

COSTA, C. et al. Toxicity in aquatic environments: discussion and evaluation methods. **Quim. Nova,** v. 31, n. 7, p. 1820-1830, 2008.

DA COSTA CUNHA, G.; ROMÃO, L. P. C.; MACEDO, Z. S. Production of alpha-alumina nanoparticles using aquatic humic substances. **Powder Technology,** v. 254, p. 344-351, 2014/03/01/ 2014. ISSN 0032-5910. Available at: < http://www.sciencedirect.com/science/article/pii/S0032591014000175 >.

DERFUS, A. M.; CHAN, W. C. W.; BHATIA, S. N. Probing the Cytotoxicity Of Semiconductor Quantum Dots. **Nano Lett,** v. 4, n. 1, p. 11-18, Jan 2004. ISSN 1530-6992. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/28890669 >.

DIEGOLI, S. et al. Interaction between manufactured gold nanoparticles and naturally occurring organic macromolecules. **Science of The Total Environment,** v. 402, n. 1, p. 51-61, 2008/08/25/ 2008. ISSN 0048-9697. Available at: < http://www.sciencedirect.com/science/article/pii/S0048969708004567 >.

DODDS, W. K. **Fresh Water Ecology: Concepts and Environmental Applications**. 1st. Academic Press, 2002. ISBN 978-0-12-219135-0.

DOMINGOS, R. F.; FRANCO, C.; PINHEIRO, J. P. Stability of core/shell quantum dots—role of pH and small organic ligands. **Environmental Science and Pollution Research,** v. 20, n. 7, p. 4872-4880, 2013/07/01 2013. ISSN 1614-7499. Available at: < https://doi.org/10.1007/s11356-012-1457-0 >.

DOMINGOS, R. F. et al. Metals in the Aquatic Environment—Interactions and Implications for the Speciation and Bioavailability: A Critical Overview. **Aquatic Geochemistry,** v. 21, n. 2, p. 231-257, 2015/07/01 2015. ISSN 1573-1421. Available at: < https://doi.org/10.1007/s10498-014-9251-x >.

DOMINGOS, R. F.; PEYROT, C.; WILKINSON, K. J. Aggregation of titanium dioxide nanoparticles: role of calcium and phosphate. **Environmental Chemistry,** v. 7, n. 1, p. 61-66, 2010. Available at: < https://doi.org/10.1071/EN09110 >.

DOMINGOS, R. F. et al. Agglomeration and dissolution of zinc oxide nanoparticles: role of pH, ionic strength and fulvic acid. **Environmental Chemistry,** v. 10, n. 4, p. 306-312, 2013. Available at: < https://doi.org/10.1071/EN12202 >.

DOMINGOS, R. F.; TUFENKJI, N.; WILKINSON, K. J. Aggregation of Titanium Dioxide Nanoparticles: Role of a Fulvic Acid. **Environmental Science & Technology,** v. 43, n. 5, p. 1282-1286, 2009/03/01 2009. ISSN 0013-936X. Available at: < https://doi.org/10.1021/es8023594 >.

DONG, H.; LO, I. M. C. Influence of calcium ions on the colloidal stability of surface-modified nano zero-valent iron in the absence or presence of humic acid. **Water Research,** v. 47, n. 7, p. 2489-2496, 2013/05/01/ 2013. ISSN 0043-1354. Available at: < http://www.sciencedirect.com/science/article/pii/S0043135413001152 >.

ELMOOR-LOUREIRO, L. M. A. **Manual de identificação de cladóceros límnicos do Brasil**. Brasilia: Universa, 1997.

EPA, U. **Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms**. Washington DC 2002.

_____. **Technical Fact Sheet – Nanomaterials**. AGENCY, E. P. United States 2017.

FABREGA, J. et al. Silver nanoparticles: Behaviour and effects in the aquatic environment. **Environment International,** v. 37, n. 2, p. 517-531, FEB 2011 2011. ISSN 0160-4120.

FARRE, M.; SANCHIS, J.; BARCELO, D. Analysis and assessment of the occurrence, the fate and the behavior of nanomaterials in the environment. **Trac-Trends in Analytical Chemistry,** v. 30, n. 3, p. 517-527, MAR 2011 2011. ISSN 0165-9936.

FERRY, J. L. et al. Transfer of gold nanoparticles from the water column to the estuarine food web. **Nat Nanotechnol,** v. 4, n. 7, p. 441-4, Jul 2009. ISSN 1748-3395. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/19581897 >.

FOLDBJERG, R. et al. PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. **Toxicology Letters,** v. 190, n. 2, p. 156-162, 2009/10/28/ 2009. ISSN 0378-4274. Available at: < http://www.sciencedirect.com/science/article/pii/S0378427409012211 >.

GAO, J. et al. Dispersion and Toxicity of Selected Manufactured Nanomaterials in Natural River Water Samples: Effects of Water Chemical Composition. **Environmental Science & Technology,** v. 43, n. 9, p. 3322-3328, 2009/05/01 2009. ISSN 0013-936X. Available at: < https://doi.org/10.1021/es803315v >.

GRILLO, R.; ROSA, A. H.; FRACETO, L. F. Engineered nanoparticles and organic matter: A review of the state-of-the-art. **Chemosphere,** v. 119, p. 608-619, 2015/01/01/ 2015. ISSN 0045-6535. Available at: < http://www.sciencedirect.com/science/article/pii/S0045653514009126 >.

GUNSOLUS, I. L. et al. Effects of Humic and Fulvic Acids on Silver Nanoparticle Stability, Dissolution, and Toxicity. **Environmental Science & Technology,** v. 49, n.

13, p. 8078-8086, 2015/07/07 2015. ISSN 0013-936X. Available at: < https://doi.org/10.1021/acs.est.5b01496 >.

HAMILTON, M.; RUSSO, R.; THURSTON, R. **TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS**. U.S. Environmental Protection Agency. Washington, D.C. 1977

HANDY, R. D. et al. Ecotoxicity test methods for engineered nanomaterials: Practical experiences and recommendations from the bench. **Environmental Toxicology and Chemistry,** v. 31, n. 1, p. 15-31, 2012/01/01 2012. ISSN 0730-7268. Available at: < https://doi.org/10.1002/etc.706 >. Accessed on: 2020/03/21.

HEINLAAN, M. et al. Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus. **Chemosphere,** v. 71, n. 7, p. 1308-16, Apr 2008. ISSN 0045-6535. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/18194809 >.

HIRIART-BAER, V. P. et al. Toxicity of silver to two freshwater algae, Chlamydomonas reinhardtii and Pseudokirchneriella subcapitata, grown under continuous culture conditions: Influence of thiosulphate. **Aquatic Toxicology,** v. 78, n. 2, p. 136-148, 2006/06/15/ 2006. ISSN 0166-445X. Available at: < http://www.sciencedirect.com/science/article/pii/S0166445X06000877 >.

HOFFMAN, D. J. et al. **Handbook of Ecotoxicology**. 1st. 1995. 768 ISBN 978-0873715850.

HOTZE, E. M.; BOTTERO, J.-Y.; WIESNER, M. R. Theoretical Framework for Nanoparticle Reactivity as a Function of Aggregation State. **Langmuir,** v. 26, n. 13, p. 11170-11175, 2010/07/06 2010. ISSN 0743-7463. Available at: < https://doi.org/10.1021/la9046963 >.

HUANG, B.; LI, D.; YANG, Y. Joint Toxicity of Two Phthalates with Waterborne Copper to Daphnia magna and Photobacterium phosphoreum. **Bulletin of Environmental Contamination and Toxicology,** v. 97, n. 3, p. 380-386, 2016/09/01 2016. ISSN 1432-0800. Available at: < https://doi.org/10.1007/s00128-016-1879-3 >.

HUND-RINKE, K. et al. Regulatory ecotoxicity testing of nanomaterials – proposed modifications of OECD test guidelines based on laboratory experience with silver and titanium dioxide nanoparticles. **Nanotoxicology,** v. 10, n. 10, p. 1442-1447, 2016/11/25 2016. ISSN 1743-5390. Available at: < https://doi.org/10.1080/17435390.2016.1229517 >.

JONATHAN, B. et al. Nanoparticle Transport, Aggregation, and Deposition. In: (Ed.). New York: McGraw-Hill Education, 2007.

JU-NAM, Y.; LEAD, J. Manufactured nanoparticles: An overview of their chemistry, interactions and potential environmental implications. **Science of the Total Environment,** v. 400, n. 1-3, p. 396-414, AUG 1 2008 2008. ISSN 0048-9697.

KAEGI, R. et al. Fate and transformation of silver nanoparticles in urban wastewater systems. **Water Res,** v. 47, n. 12, p. 3866-77, Aug 2013. ISSN 1879-2448. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/23571111 >.

KALMAN, J. et al. Characterisation of bioaccumulation dynamics of three differently coated silver nanoparticles and aqueous silver in a simple freshwater food chain. **Environmental Chemistry,** v. 12, n. 6, p. 662-672, 2015.

KIM, J. S. et al. Antimicrobial effects of silver nanoparticles. **Nanomedicine: Nanotechnology, Biology and Medicine,** v. 3, n. 1, p. 95-101, 2007/03/01/ 2007. ISSN 1549-9634. Available at: < http://www.sciencedirect.com/science/article/pii/S1549963406003467 >.

KINNIBURGH, D. G. **FIT User Guide, Technical Report WD/93/23.** Keyworth: UK: British Geological Survey 1993.

KLAINE, S. J. et al. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. **Environ Toxicol Chem,** v. 27, n. 9, p. 1825-51, Sep 2008. ISSN 0730-7268. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/19086204 >.

KNAUER, K.; BUFFLE, J. ADSORPTION OF FULVIC ACID ON ALGAL SURFACES AND ITS EFFECT ON CARBON UPTAKE. **Journal of Phycology,** v. 37, n. 1, p. 47-51, 2001/02/06 2001. ISSN 0022-3646. Available at: < https://doi.org/10.1046/j.1529-8817.2001.037001047.x >. Accessed on: 2020/03/21.

KOUKAL, B. et al. Effect of Pseudokirchneriella subcapitata (Chlorophyceae) exudates on metal toxicity and colloid aggregation. **Water Research,** v. 41, n. 1, p. 63-70, 2007/01/01/ 2007. ISSN 0043-1354. Available at: < http://www.sciencedirect.com/science/article/pii/S0043135406005331 >.

LABILLE, J.; BRANT, J. Stability of nanoparticles in water. **Nanomedicine (Lond),** v. 5, n. 6, p. 985-98, Aug 2010. ISSN 1748-6963. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/20735232 >.

LAPRESTA-FERNÁNDEZ, A.; FERNÁNDEZ, A.; BLASCO, J. Nanoecotoxicity effects of engineered silver and gold nanoparticles in aquatic organisms. **TrAC Trends in Analytical Chemistry,** v. 32, p. 40-59, 2012/02/01/ 2012. ISSN 0165-9936. Available at: < http://www.sciencedirect.com/science/article/pii/S0165993611003748 >.

LI, M. et al. Stability, Bioavailability, and Bacterial Toxicity of ZnO and Iron-Doped ZnO Nanoparticles in Aquatic Media. **Environmental Science & Technology,** v. 45, n. 2, p. 755-761, 2011/01/15 2011. ISSN 0013-936X. Available at: < https://doi.org/10.1021/es102266g >.

LIU, H.; FANG, H. H. P. Characterization of electrostatic binding sites of extracellular polymers by linear programming analysis of titration data. **Biotechnology and Bioengineering,** v. 80, n. 7, p. 806-811, 2002/12/30 2002. ISSN 0006-3592. Available at: < https://doi.org/10.1002/bit.10432 >. Accessed on: 2020/03/19.

LIU, J.; HURT, R. H. Ion Release Kinetics and Particle Persistence in Aqueous Nano-Silver Colloids. **Environmental Science & Technology,** v. 44, n. 6, p. 2169-2175, 2010/03/15 2010. ISSN 0013-936X. Available at: < https://doi.org/10.1021/es9035557 >.

LOK, C. N. et al. Silver nanoparticles: partial oxidation and antibacterial activities. **J Biol Inorg Chem,** v. 12, n. 4, p. 527-34, May 2007. ISSN 0949-8257. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/17353996 >.

MACCARTHY, P. A. M. R. L. A. C. C. E. A. B. P. R. An Introduction to Soil Humic Substances. p. 1-12, 1990. ISSN 9780891188742. Available at: < https://onlinelibrary.wiley.com/doi/abs/10.2136/1990.humicsubstances.c1 >.

MANAHAN, S. E. **Environmental chemistry**. 8th. 2005. ISBN 1-56670-633-5.

MAURER-JONES, M. A. et al. Toxicity of Engineered Nanoparticles in the Environment. **Analytical Chemistry,** v. 85, n. 6, p. 3036-3049, 2013/03/19 2013. ISSN 0003-2700. Available at: < https://doi.org/10.1021/ac303636s >.

MENSCH, A. C. et al. Natural Organic Matter Concentration Impacts the Interaction of Functionalized Diamond Nanoparticles with Model and Actual Bacterial Membranes. **Environ Sci Technol,** v. 51, n. 19, p. 11075-11084, Oct 2017. ISSN 1520-5851. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/28817268 >.

MIELKE, R. E. et al. Differential growth of and nanoscale TiO_2 accumulation in Tetrahymena thermophila by direct feeding versus trophic transfer from Pseudomonas aeruginosa. **Applied and environmental microbiology,** v. 79, n. 18, p. 5616-5624, 2013. ISSN 1098-5336
0099-2240. Available at: < https://pubmed.ncbi.nlm.nih.gov/23851096 >.Available at: < https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3754167/ >.

MILNE, C. J.; KINNIBURGH, D. G.; TIPPING, E. Generic NICA-Donnan Model Parameters for Proton Binding by Humic Substances. **Environmental Science & Technology,** v. 35, n. 10, p. 2049-2059, 2001/05/01 2001. ISSN 0013-936X. Available at: < https://doi.org/10.1021/es000123j >.

MIURA, N.; SHINOHARA, Y. Cytotoxic effect and apoptosis induction by silver nanoparticles in HeLa cells. **Biochemical and Biophysical Research Communications,** v. 390, n. 3, p. 733-737, 2009/12/18/ 2009. ISSN 0006-291X. Available at: < http://www.sciencedirect.com/science/article/pii/S0006291X09020038 >.

NAVARRO, E. et al. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. **Ecotoxicology,** v. 17, n. 5, p. 372-386, 2008/07/01 2008. ISSN 1573-3017. Available at: < https://doi.org/10.1007/s10646-008-0214-0 >.

NIKINMAA, M. **An Introduction to Aquatic Toxicology**. Elsevier Science, 2014. ISBN 9780124115811. Available at: < https://books.google.com.br/books?id=dQJ0AwAAQBAJ >.

OECD. **Test No. 202: Daphnia sp. Acute Immobilisation Test**. 2004. Available at: < https://www.oecd-ilibrary.org/content/publication/9789264069947-en >.

_____. **Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test**. 2011. Available at: < https://www.oecd-ilibrary.org/content/publication/9789264069923-en >.

_____. **Test No. 211: Daphnia magna Reproduction Test**. 2012. Available at: < https://www.oecd-ilibrary.org/content/publication/9789264185203-en >.

PANYALA, R. N.; PENA-MENDEZ, M. E.; HAVEL, J. Silver or silver nanoparticles: a hazardous threat to the environment and human health? **Journal of Applied Biomedicine,** v. 6, n. 3, p. 117-129, / 2008. ISSN 1214021X. Available at: < https://jab.zsf.jcu.cz/artkey/jab-200803-0001.php >.Available at: < http://dx.doi.org/10.32725/jab.2008.015 >.

PICCINNO, F. et al. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. **Journal of Nanoparticle Research,** v. 14, n. 9, p. 1109, 2012/08/19 2012. ISSN 1572-896X. Available at: < https://doi.org/10.1007/s11051-012-1109-9 >.

RATTE, H. T. Bioaccumulation and toxicity of silver compounds: A review. **Environmental Toxicology and Chemistry,** v. 18, n. 1, p. 89-108, 1999/01/01 1999. ISSN 0730-7268. Available at: < https://doi.org/10.1002/etc.5620180112 >. Accessed on: 2019/09/30.

REYNOLDS, C. S. **The Ecology of Freshwater Phytoplankton**. Cambridge University Press, 1984. 396 ISBN 978-0521282222.

RIBEIRO, F. et al. Silver nanoparticles and silver nitrate induce high toxicity to Pseudokirchneriella subcapitata, Daphnia magna and Danio rerio. **Science of The Total Environment,** v. 466-467, p. 232-241, 2014/01/01/ 2014. ISSN 0048-9697. Available at: < http://www.sciencedirect.com/science/article/pii/S0048969713007535 >.

_____. Bioaccumulation of silver in Daphnia magna: Waterborne and dietary exposure to nanoparticles and dissolved silver. **Science of The Total Environment,** v. 574, p. 1633-1639, 2017/01/01/ 2017. ISSN 0048-9697. Available at: < http://www.sciencedirect.com/science/article/pii/S0048969716319076 >.

ROCHA, J. C.; ROSA, A. H. **Substâncias húmicas aquáticas Interação com espécies metálicas**. 1. 2003. 126 ISBN 8571394741.

RODGHER, S. et al. Change in life cycle parameters and feeding rate of Ceriodaphnia silvestrii Daday (Crustacea, Cladocera) exposure to dietary copper. **Ecotoxicology,** v. 17, n. 8, p. 826-833, 2008/11/01 2008. ISSN 1573-3017. Available at: < https://doi.org/10.1007/s10646-008-0245-6 >.

ROSA, A. H. et al. Estudo da labilidade de Cu(II), Cd(II), Mn(II) e Ni(II) em substâncias húmicas aquáticas utilizando-se membranas celulósicas organomodificadas. **Química Nova,** v. 30, p. 59-65, 2007. ISSN 0100-4042. Available at: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-40422007000100014&nrm=iso >.

SANTOS, A. C. et al. Characterization of interactions between natural organic matter and metals by tangential-flow ultrafiltration and ICP OES. **Journal of the Brazilian Chemical Society,** v. 22, p. 98-103, 2011. ISSN 0103-5053. Available at: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0103-50532011000100013&nrm=iso >.

SANTOS, M. A. P. F.; VICENSOTTI, J.; MONTEIRO, R. **Sensitivity of Four Test Organisms (Chironomus xanthus, Daphnia magna, Hydra attenuata and Pseudokirchneriella subcapitata) to NaCl: an Alternative Reference Toxicant**. Journal of the Brazilian Society of Ecotoxicology. 2**:** 229-236 p. 2007.

SEDERS, L. A.; FEIN, J. B. Proton binding of bacterial exudates determined through potentiometric titrations. **Chemical Geology,** v. 285, n. 1, p. 115 - 123, 2011. ISSN 0009-2541. Available at: < http://www.sciencedirect.com/science/article/pii/S0009254111001458 >.

SHARMA, V. K. et al. Organic-coated silver nanoparticles in biological and environmental conditions: Fate, stability and toxicity. **Advances in Colloid and Interface Science,** v. 204, p. 15-34, 2014/02/01/ 2014. ISSN 0001-8686. Available at: < http://www.sciencedirect.com/science/article/pii/S0001868613001735 >.

SHI, Z.; NURMI, J. T.; TRATNYEK, P. G. Effects of nano zero-valent iron on oxidation-reduction potential. **Environ Sci Technol,** v. 45, n. 4, p. 1586-92, Feb 2011. ISSN 1520-5851. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/21204580 >.

SIKORA, F. J.; STEVENSON, F. J. Silver complexation by humic substances: Conditional stability constants and nature of reactive sites. **Geoderma,** v. 42, n. 3, p. 353-363, 1988/08/01/ 1988. ISSN 0016-7061. Available at: < http://www.sciencedirect.com/science/article/pii/0016706188900110 >.

STANDARDIZATION, I. I. O. F. **Water quality — Fresh water algal growth inhibition test with unicellular green algae**. 8692:2012 2012.

STEVENSON, F. J. **Humus Chemistry: Genesis, Composition, Reactions**. Wiley, 1994. ISBN 9780471594741. Available at: < https://books.google.com.br/books?id=7kCQch_YKoMC >.

SUN, X.-F. et al. Spectroscopic study of Zn2+ and Co2+ binding to extracellular polymeric substances (EPS) from aerobic granules. **Journal of Colloid and Interface Science,** v. 335, n. 1, p. 11-17, 2009/07/01/ 2009. ISSN 0021-9797. Available at: < http://www.sciencedirect.com/science/article/pii/S0021979709004251 >.

TERKHI, M. C. et al. Fourier transform infrared study of mercury interaction with carboxyl groups in humic acids. **Journal of Photochemistry and Photobiology A: Chemistry,** v. 198, n. 2, p. 205-214, 2008/08/15/ 2008. ISSN 1010-6030. Available at: < http://www.sciencedirect.com/science/article/pii/S1010603008001391 >.

THARAUD, M. et al. uFREASI: user-FRiendly Elemental dAta procesSIng. A free and easy-to-use tool for elemental data treatment. **Microchemical Journal,** v. 121, p. 32-40, 2015/07/01/ 2015. ISSN 0026-265X. Available at: < http://www.sciencedirect.com/science/article/pii/S0026265X15000132 >.

THURMAN, E. M.; MALCOLM, R. L. Preparative isolation of aquatic humic substances. **Environmental Science & Technology,** v. 15, n. 4, p. 463-466, 1981/04/01 1981. ISSN 0013-936X. Available at: < https://doi.org/10.1021/es00086a012 >.

TONIETTO, A. Cylindrospermopsis raciborskii (Cyanobacteria) exudates: Chemical characterization and complexation capacity for Cu, Zn, Cd and Pb. **Water Research,** v. 49, p. 381 - 390, 2014. ISSN 0043-1354. Available at: < http://www.sciencedirect.com/science/article/pii/S0043135413008063 >.

USEPA. **Acid Digestion Of Waters For Total Recoverable Or Dissolved Metals For Analysis By FLAA Or ICP Spectroscopy**. METHOD 3005A**:** 5 p. 1992.

_____. **Method 3050B: Acid Digestion of Sediments, Sludges, and Soils**. Washington, DC 1996.

_____. **Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms**. Washington DC 2002.

VERMEER, A. W. P. **Interactions between humic acid and hematite and their effects on metal ion speciation**. 1996. Vermeer, S.l.

WAGNER, S. A. G. A. A. N. E. A. H. T. A. V. D. K. F. Spot the Difference: Engineered and Natural Nanoparticles in the Environment—Release, Behavior, and Fate. **Angewandte Chemie International Edition,** v. 53, n. 46, p. 12398-12419, 2014. Available at: < https://onlinelibrary.wiley.com/doi/abs/10.1002/anie.201405050 >.

WEST, I.; GULLEY, D.: University of Wyoming 1996.

WETZEL, R. G. **Limnology**. 3rd. Academic Press, 2001. 1006 ISBN 978-0-12-744760-5.

WIESNER, M.; BOTTERO, J. Y. **Environmental Nanotechnology : Applications and Impacts of Nanomaterials: Applications and Impacts of Nanomaterials**. Mcgraw-hill, 2007. ISBN 9780071477505. Available at: < https://books.google.com.br/books?id=zsH_emlfJ9wC >.

YANG, Y. et al. Analysis of silver and gold nanoparticles in environmental water using single particle-inductively coupled plasma-mass spectrometry. **Science of The Total Environment,** v. 563-564, p. 996-1007, 2016/09/01/ 2016. ISSN 0048-9697.

Available at: < http://www.sciencedirect.com/science/article/pii/S004896971531305X >.

ZAGATTO, P. A.; BERTOLETTI, E. **Ecotoxicologia Aquática - Princípios e Aplicações**. 2006. 464.

ZENG, J. et al. Composition and aggregation of extracellular polymeric substances (EPS) in hyperhaline and municipal wastewater treatment plants. **Scientific Reports,** v. 6, n. 1, p. 26721, 2016/05/25 2016. ISSN 2045-2322. Available at: < https://doi.org/10.1038/srep26721 >.

ZHANG, L. et al. Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). **Journal of Nanoparticle Research,** v. 9, n. 3, p. 479-489, 2007. Available at: < https://www.scopus.com/inward/record.uri?eid=2-s2.0-34248343855&doi=10.1007%2fs11051-006-9150-1&partnerID=40&md5=978007502c1c2527fa1b93e49b67db98 >.

ZHAO, C.-M.; WANG, W.-X. Comparison of acute and chronic toxicity of silver nanoparticles and silver nitrate to Daphnia magna. **Environmental Toxicology and Chemistry,** v. 30, n. 4, p. 885-892, 2011/04/01 2011. ISSN 0730-7268. Available at: < https://doi.org/10.1002/etc.451 >. Accessed on: 2019/07/17.

_____. Size-Dependent Uptake of Silver Nanoparticles in Daphnia magna. **Environmental Science & Technology,** v. 46, n. 20, p. 11345-11351, 2012/10/16 2012. ISSN 0013-936X. Available at: < https://doi.org/10.1021/es3014375 >.

ZHENG, S. et al. Role of extracellular polymeric substances on the behavior and toxicity of silver nanoparticles and ions to green algae Chlorella vulgaris. **Science of The Total Environment,** v. 660, p. 1182-1190, 2019/04/10/ 2019. ISSN 0048-9697. Available at: < http://www.sciencedirect.com/science/article/pii/S0048969719300737 >.

ZHOU, K. et al. The role of exopolymeric substances in the bioaccumulation and toxicity of Ag nanoparticles to algae. **Scientific Reports,** v. 6, n. 1, p. 32998, 2016/09/12 2016. ISSN 2045-2322. Available at: < https://doi.org/10.1038/srep32998 >.

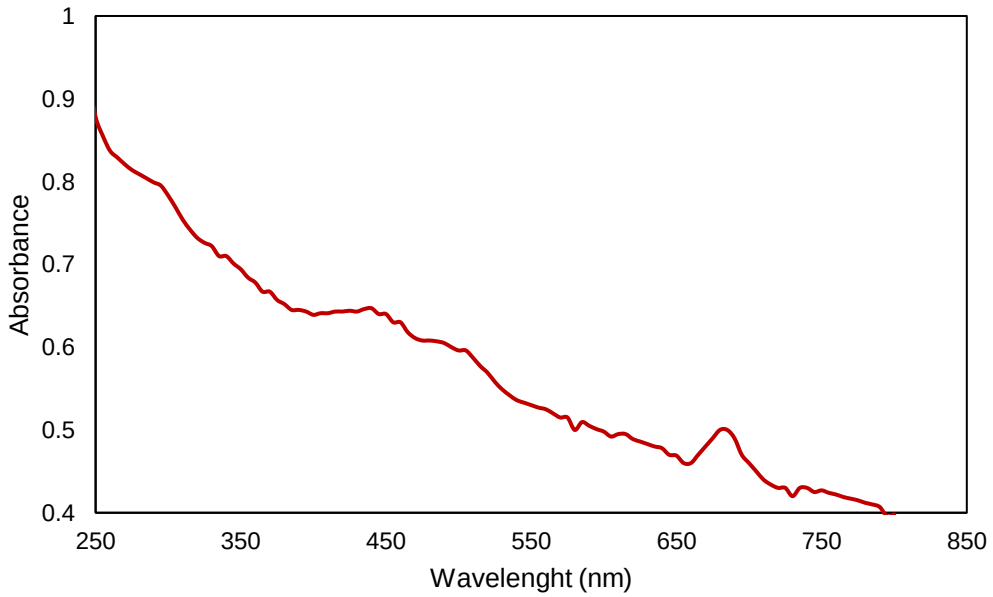
ZHU, X. et al. Trophic transfer of TiO2 nanoparticles from daphnia to zebrafish in a simplified freshwater food chain. **Chemosphere,** v. 79, n. 9, p. 928-933, 2010/05/01/ 2010. ISSN 0045-6535. Available at: < http://www.sciencedirect.com/science/article/pii/S004565351000305X >.

Appendix 1 -Study envolving cells counting by spectrophotometry.

As an alternative method to obtain the algae concentration after maximization process, the spectrophotometry UV-VIS was used. The method consists in obtain a relation between the absorbance value *versus* cellular concentration (cell mL^{-1}) based on the method proposed by Souza (2012). The author proposes a counting relation based on the absorbance value in order to facilitate the counting procedure due to time demand, chambers and/or specific microscopes to obtain the concentration.

The selection of the working wavelength was performed using a Varian spectrophotometer, for scanning along the wavelengths in the region between 200 and 800 nm using an algal solution of *R. subcapitata* in the concentration of 10^7 cells mL^{-1}, previously determined using a counting chamber (Fuchs-Rosenthal chamber). According to the spectrum obtained (Figure A1) the working wavelength of 685 nm was chosen for algae concentration, the same wavelength determined by Souza (2012).

Figure A1 – Absorbance obtained by Varian spectrophotometer.



The results obtained by the relation between absorbance and algal concentration using linear regression were organized as graphs to obtain the calibration curve in two ways as shown in Figure A2. The first curve (Figure A2 a) was constructed with all the concentration measurement points. In contrast, two distribution

point curves of lower concentration values were plotted at a minimum of 5 points (Figure A2b) and higher concentrations as seen in Figure A2c.

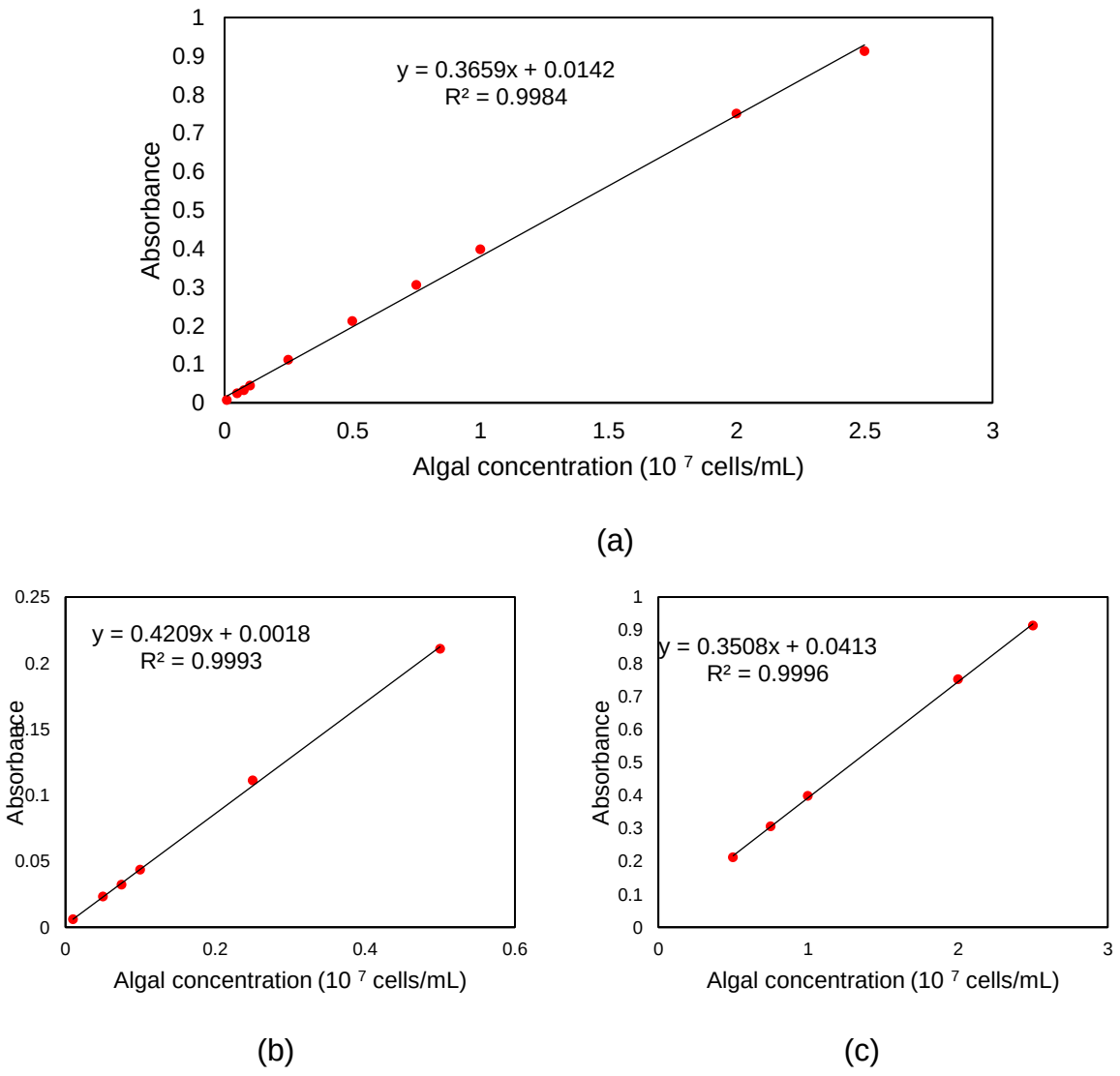


Figure A2– Calibration curve using cell concentration and absorbance values measured in Femto Spectrophotometer (wavelength 685 nm). a) curve containing all measured points b) curve with low concentrations (0.01 to 0.5 x 10^7 cells mL^{-1}) c) curve with high concentrations (0.5 to 2.5 x 10^7 cells mL^{-1}).

Thus, by checking the correlation coefficient obtained through the equation of the line obtained by constructing a linear curve, it is possible to observe the best coefficients and, consequently, the best curves are those separated in two concentration curves. Souza (2012), also compares a construction of two curves and observes the same behavior. To do so, the use of Absorbance and concentration ratios for a growth curve construct of *R. subcapitata* was performed, to optimize counting of

homogeneous algae solution as (e.g. internal production and control of cultures, as food for organism like *D. similis*, also cultivated at this laboratory).

SOUZA, M.B. (Dissertação). Avaliação da toxicidade aguda de um herbicida comercial e dos componentes químicos Diuron e Hexazinona em *Ceriodaphnia dubia*. Universidade de Ribeirão Preto – UNAERP. 2012

Appendix 2 – Oligo Medium preparation

All the flasks, instruments and medium solution used in the experiments were previously sterilized. The procedures for sterilization are briefly described.

Materials: - 7 solutions according to the Table A1; volumetric flask (1L); ultrapure water; 7 autoclaved tips; micropipettes; erlenmeyer, ethanol 70 % (v/v).

Table A1 - LC Oligo Medium composition. Source: ABNT (2018).

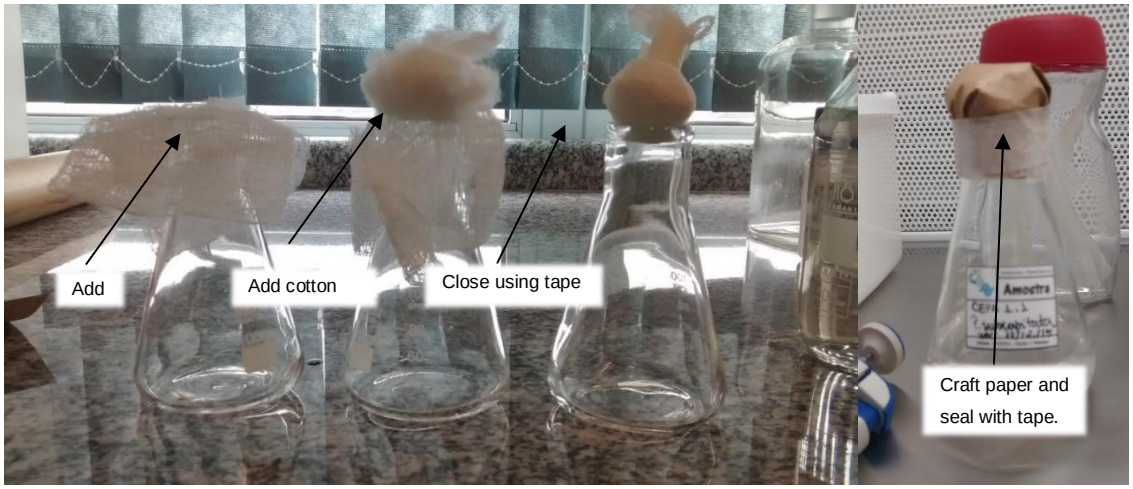
Solution	Reagent	Mass/mg	Preparation	Volume*/mL
1	$Ca(NO_3)_2$. $4H_2O$	4.0	To dissolve and complete to 100 mL in ultrapure water	1.0
2	KNO_3	10.0	To dissolve and complete to 100 mL in ultrapure water	1.0
3	$MgSO_4.7H_2O$	3.0	To dissolve and complete to 100 mL in ultrapure water	1.0
4	K_2HPO_4	4.0	To dissolve and complete to 100 mL in ultrapure water	1.0
5	$CuSO_4.5H_2O$	0.03	To dissolve and complete to 100 mL in ultrapure water	0.5
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.06		
	$ZnSO_4.7H_2O$	0.06		
	$CoCl_2.6H_2O$	0.06		
	$Mn(NO_3)_2.4H_2O$	0.06		
	$C_6H_8O_2.H_2O$	0.06		
	H_3BO_3	0.06		
6	$C_6H_5FeO_7.5H_2O$	1.625	To dissolve and complete to 100 mL in ultrapure water	0.5
	$FeCl_3.6H_2O$	0.625		
	$FeSO_4$. $7H_2O$	0.625		
7	$NaHCO_3$	15	To dissolve and complete to 100 mL in ultrapure water	1.0

* Volume (mL) to prepare 1 liter L.C. Oligo medium.

Adjust pH, stirrer, sterilize using autoclave at 121 ºC for 15 minutes.

- The ratio of medium to total volume Erlenmeyer is 1: 3. This means that in an erlenmeyer of about 100 mL, 30-40 mL of medium is placed.

Cotton plug preparation for erlenmeyers:



Conceal or place in a bowl 7 pipettes for autoclaving.

To proceed with the maximization:

1. Separate the appropriate PPE, observing the photo: cap, glasses, mask, gloves (special item)

Obs: Use the materials only at the moment you start the maximization procedure.

2. Separate the material that will be used inside the laminar flow chamber.

3. Switch on general switch, connect motor and switch on UV light;

Note: When UV (ultraviolet light) is on, keep the warning and plate in sight of other people and avoid entering the microbiology laboratory.

4. Leave the UV light on for about 10-20 minutes and turn it off. After the time has elapsed, switch off the UV light switch and turn on the normal light.
5. Make the inoculum, and then identify the repiques with the date of inoculation and the name of the organism.
6. Place in incubator and repeat procedure every 15 days (ideally at most one month).

AgNP storage

The Figure A3 shows a glove box (left side) glove bag (right side) for AgNP stocks separation before perform the test, to avoid previous oxidation during the storage time. The glove box was manipulated according to procedures stablished and the glove bags were adapted with laboratory materials (e.g. nitrile gloves, silver tape, vacuum pump air, nitrogen cylinder, tubes for connect, bags). In order to produce an inert environment, the nitrogen was purge inside the chamber/ bag while the oxygen present was removed.

Figure A3 – Glove box (left) and glove bag (right) for AgNP storage procedure.

Appendix 3 – SOP ICPMS Thermo scientific.

ICP-MS Thermo Element

1st Part (Around 1 and ½ hour)

1. Initializing the machine

1.1 Check-the cooler temperature;
1.2 Open the gas valve (⟷);
1.3 Check gas Ar is in indicating 10 bar;
1.4 Insert the cones in them respectively position (see SM1);
1.5 Close sample door;
1.6 Lock the sample door (listen some closing sound);
1.7 Verify if the tubes are good
1.8 Verify if need Add a standard. If yes:
1.8.1 Remove the normal introduction tygon tube;
1.8.2 Change it for the tube Y (system tube with 2 input and 1 output);
1.9 Check the HNO_3 1 % (v/v) and Milli-Q bottles, Rinse 1 and Rinse 2, respectively (if isn't enough to the analyses do fill the bottles properly, see SM2);
1.10 Open the Exit air in the bottles of HNO_3 1% and Milli-Q;
1.11 Open the bottles tap;
1.12 Go to computer to proceed with the software initialization.

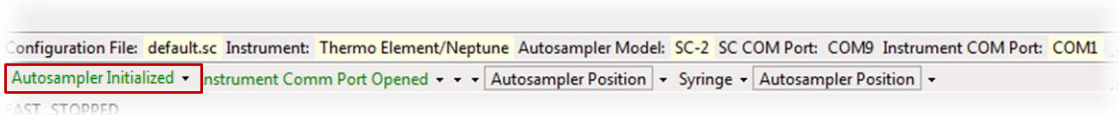
2. Initializing the software

Proceed with this steps only if the computer is turned off. If the computer is already turned on and the software already opened, go directly to the Step 3.

2.1 Turn on the computer and use the Element login user;
2.2 To open the interface equipment/ user, click on "Thermo element" ();
2.3 To turn on the auto sampler, click on "ESISC" ();
2.4 In the ESISC window, click on "Initialize" (in order to initialize the auto sampler).

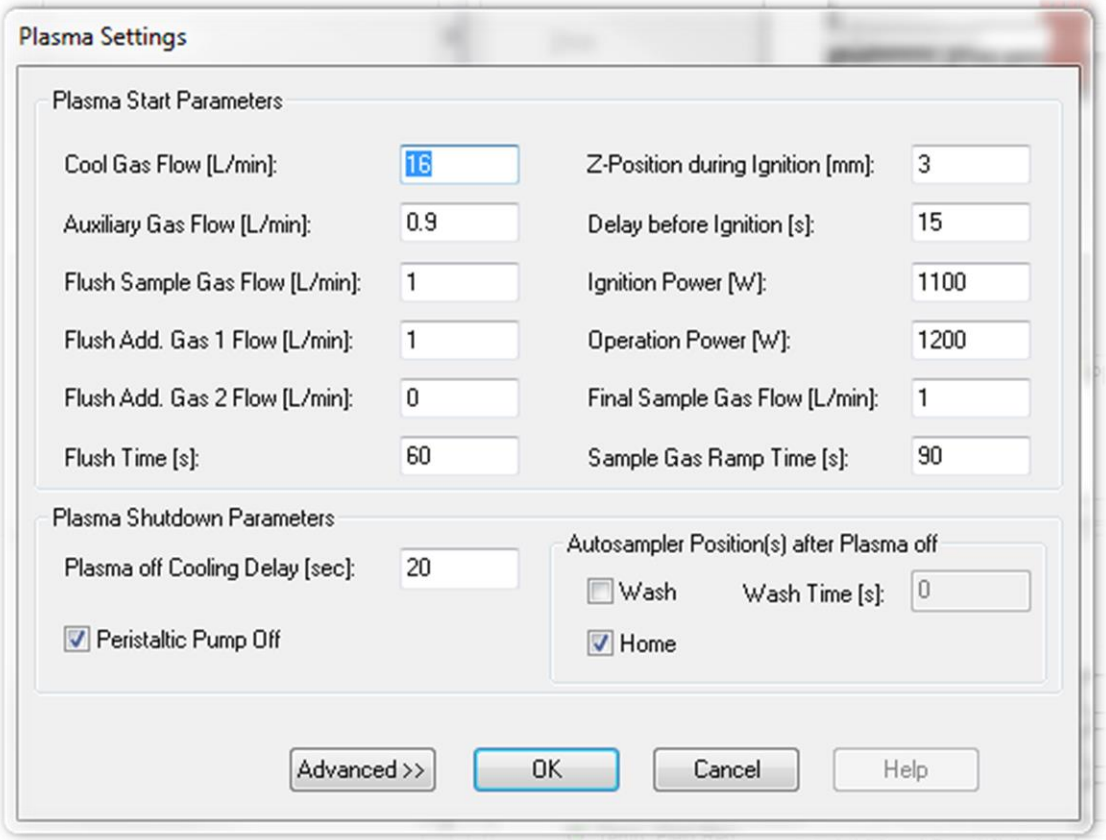
Obs1: You'll listen some noises when the auto sampler is being initialize and see the sampler-taken moving a bit.

Obs2: Never click on initialize if the auto sampler is already initialized. With a message in a box below the auto-sampler you can identify if it is already initialized.



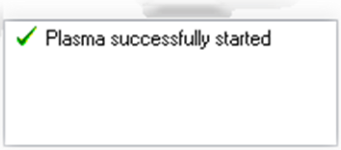
3. Turn on the plasma

3.1 On Instrument-Thermo element window, go to "Settings" in order to set the parameters, and check them:



3.2 Click on: “Ok” (the window will be closed);
3.3 To turn on the plasma, click on the button: “On”;
3.4 It will appear one window containing the pump direction, just click on: “Ok”;
3.5 Wait for each steps until appear the sequence messages:

✓ Switching cooling System ON
✓ Switch on gas flows
✓ Switching interface pump ON
✓ Initializing RF generator
✓ Ignite plasma
✓ Switch on sample gas
✓ **Plasma successfully started**



After the last message, the compartments installed will be in green color (see figure 2), if the initialization process was successfully started. If not, repeat the sequence 2 again searching for possible failure reasons.

In order to stabilize the parameters (fore vacuum ($2x10^{-4}$ →$2\ 10^{-3}$) and high vacuum (10^{-8} → 10^{-7}))

4. Rinse time during 3600 seconds:

4.1 Go to auto-sampler window “ESISC” (ESI);
4.2 Change the Rinse time for: Rinse 1: 3600 seconds
Rinse 2: 60 seconds

4.3 Click on: Rinse/Wash

5. Insert Initial parameters for Tuning

5.1 Click on “Run tune” (first icon)

5.2 In the Run tune window, check if the file “FAST.tpf” is opened;

5.3 Click on “Create scan list” (icon);

5.3.1 Click on “Load”;

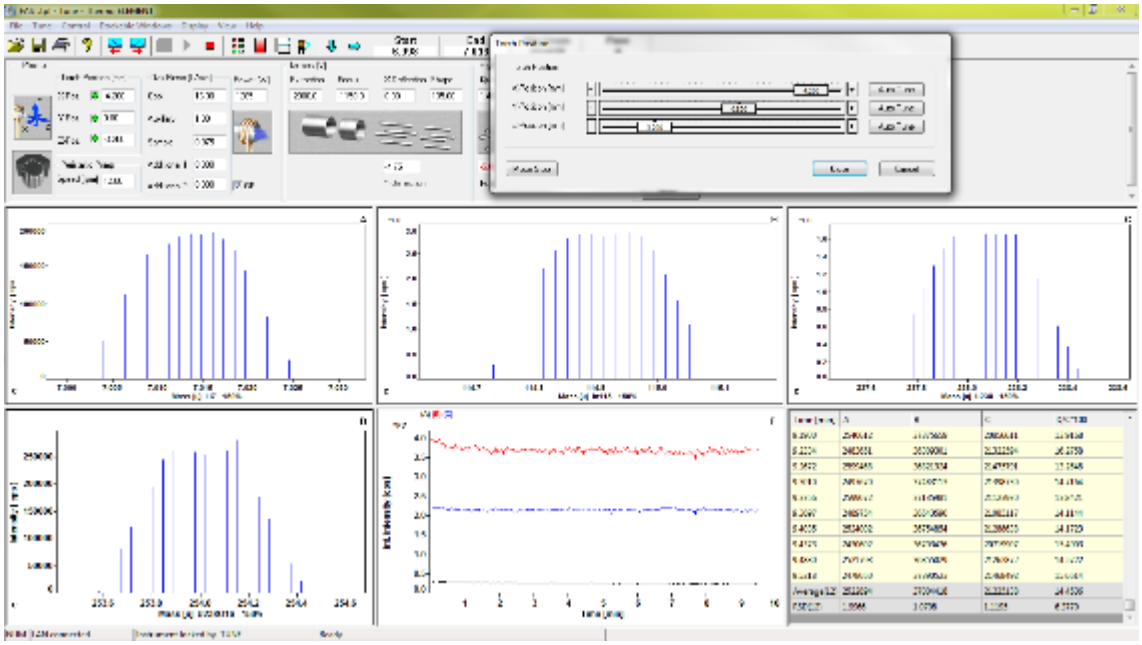
5.3.2 Load the file: Thermo_HP_TuneUo.scl

5.3.3 Open

5.3.4 Ok

5.4 Click on “Start” and wait for a few seconds (around 10 s);

5.4.1 See the 4 diagrams Li 7; In115; U 238; UOx



5.5 Click on “Stop”, to stop the scan.

5.6 Wait for around 1 hour;

5.7 Write in the book:

User: *Cláudia/ Rute*

Date: *dd/mm/yyyy* Switch-On time: *hh h mm* Switch-Off time:

NB:

Switch OK!

Tuning

Mass Calibration

Daily Test

Mass off Set

2nd Part (After 1 hour)

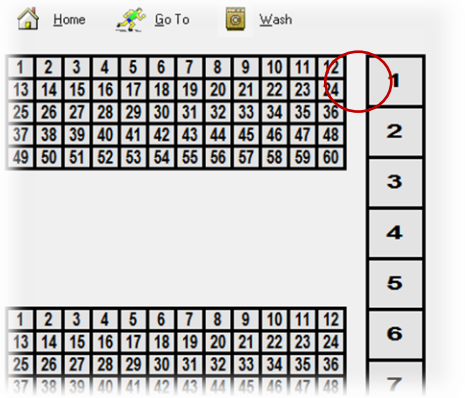
6. Tuning

6.1 Insert the Tuning Solution* in the Position 1 (Big rack);

* Tuning solution is a multi-elemental solution of 1 ppb in concentration used to adjust the stability.

6.2 Select the 1st position in the auto sampler window and “Go to” (

 );



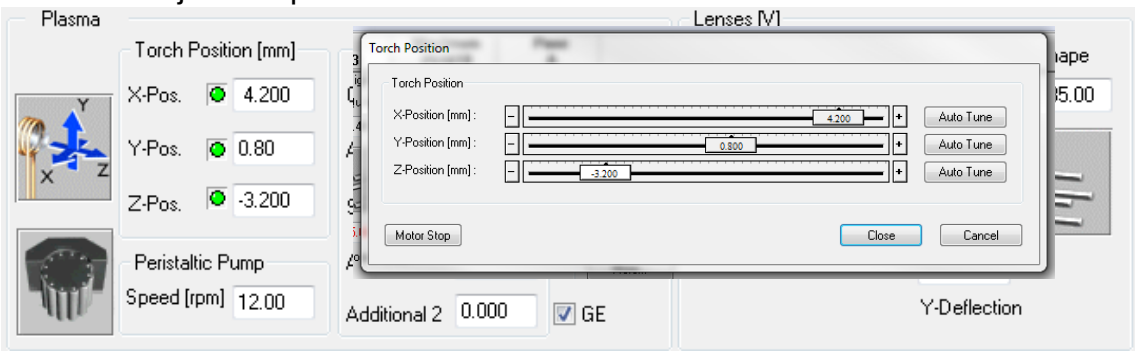
Proceed with Tuning in Low, Medium and High Resolution;

6.3 Low Resolution

Once the Load the file opened is Thermo_HP_TuneUo.scl (previously downloaded), just proceed with the Low resolution Tuning.

6.3.1 Click on “Start” ()

6.3.2 To adjust the parameters:



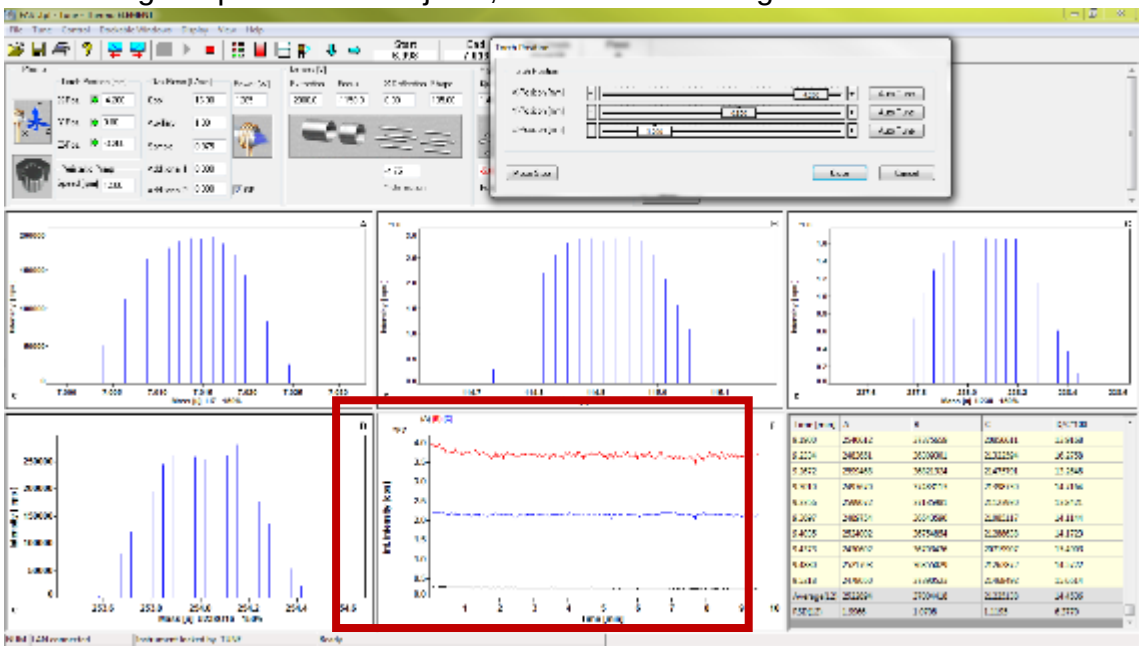
6.3.2.1 Torch position (X, Y, Z);

6.3.2.2 Plasma parameter: gas flow, samples gas.

Obs: Z, Gas flow and sample gas = adjust generally when have high production of UOx. Its’s also possible to adjust the Y and X for torch position; gas flow and sample gas for plasma parameter.

6.3.2.3 For A, B and C the RSD shouldn't be more than 1.00 %, maximum 1.2 %. Check the D/C*100 parameter (Look at the table where the average should be between 8 and 9. Finally the In signal should be higher than 10^6).

6.3.3 During the parameters adjusts, see/check the diagram with red mark:



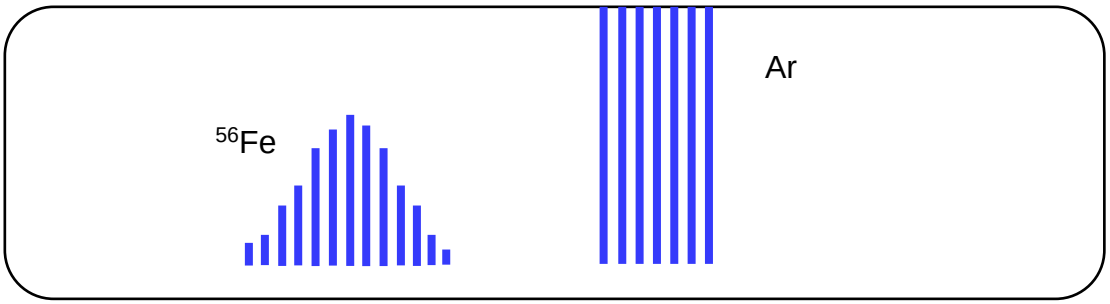
Obs: When the In115 is not added as a Internal Standard, the sequence of color is blue, red and black.

6.3.4 When all the parameters are adjusted click on "Stop Scan" (icon ■);
6.3.5 Go to Medium Resolution adjust.

6.4 <u>Medium Resolution</u>

6.4.1 Click on: Create a Scan List (icon );
6.4.2 Go to: Load Thermo_MR_TuneFeArO_Resolution.scl
6.4.3 Open
6.4.4 Ok
6.4.5 Click on "Start"
6.4.6 After some seconds, 2 peaks will appear:

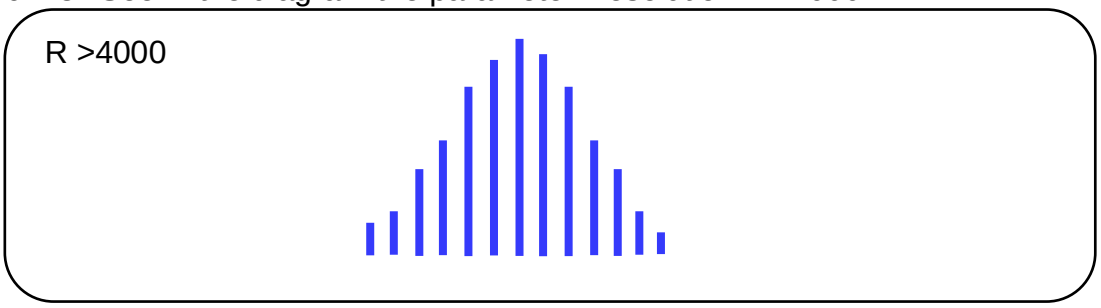
Obs: Sometimes, the 2 peaks are over load the window and need to be framed.



6.4.7 Adjust the mass range
Start: (55.8XX) End: (55.8XX)

Press: the icon “Spectrum parameter”)

6.4.8 See in the diagram the parameter Resolution = >4000

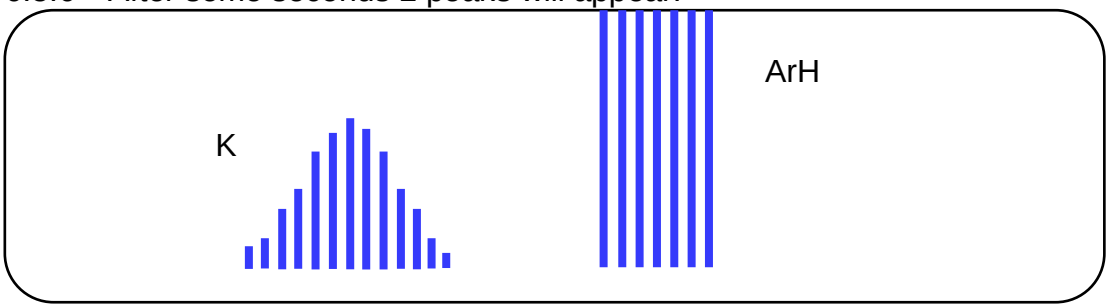


6.4.8.1 If the R isn’t “good” we can change the parameter Focus Quad
6.4.8.1.1 For change the parameter double click in The icon of Focus Quad (Focus Quad 1; Rotation Quad 1; Rotation Quad 2);
6.4.8.1.2 Change the parameter until obtain R > 4000

6.4.9 When the parameter is adjusted, click on “Stop Scan” (icon);
6.4.10 Go to High Resolution adjust.

6.5 <u>High Resolution</u>

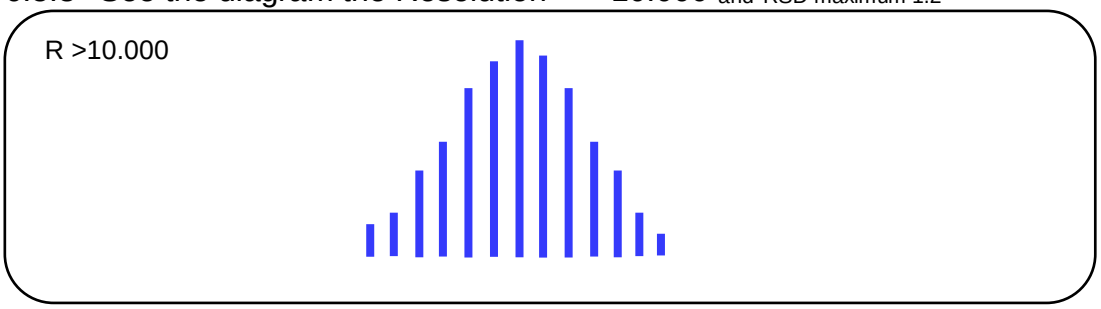
6.5.1 Click on: Create a Scan List (icon);
6.5.2 Load Thermo_HR_Tune_K_ArH_Resolution.scl
6.5.3 Open
6.5.4 Ok
6.5.5 Start scan (between 38.96 and 18.97).
6.5.6 After some seconds 2 peaks will appear:



6.5.7 Adjust the mass range
Start: (38.9XX) End: (38.9XX)

Press: the icon Set → (Spectrum parameter)

6.5.8 See the diagram the Resolution = > 10.000 and RSD maximum 1.2



6.5.8.1 If the R isn't "good" we can change the parameter Focus Quad (same from Medium Resolution)

6.5.9 Stop scan

6.5.10 Close the window "Run Tune";

6.5.11 Fill the parameters in the book:

LR
MR
HR
NB: ✓ *Switch on* ✓ *Tuning* *Mass Calibration* *Daily Test* *Mass off Set*

7. Mass Calibration (2nd Icon)

7.1 Click in: Run sequence (second icon)

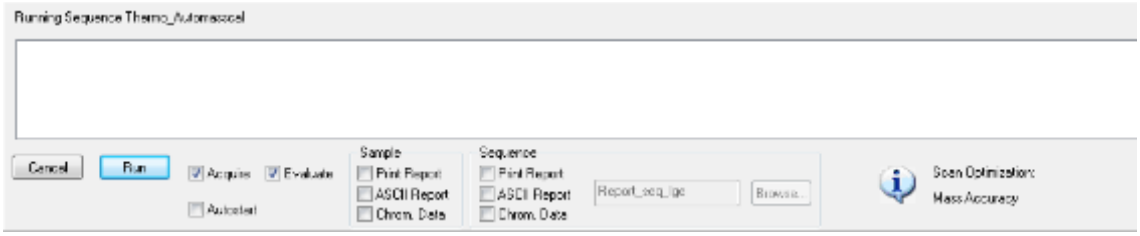
7.2 Go to: File → Open → Daily sequences → Thermo Auto MassCal.seq

7.3 Reset item (icon)→ All

7.4 Go to: Actions → Disable Auto lock mass (without tick);

7.5 Click on "Start" (icon) (One new window below the screen will appear);

7.6 Select in the window:



✓ Acquire
✓ Evaluate
7.7 Click on: “Run” (a new window will appear);
7.8 Click on: “Continue”
During the mass calibration, each step will be run:

After the Thermo_LR_Wide run had finished, don’t click on Continue;

7.9 Click on the icon in the window Instrument: “Run mass calibration” (7th icon );
7.10 Click on: File → Open → Thermo_LR_Wide (generally select the1st line and check the file time if is almost the real time sett in the equipment)
7.11 Thermo_LR_Wide

7.11.1 Click on “Manual calibration” (icon  , and one small window with the elements will appear);

7.11.2 Click on: “Zoom Next peak” (icon );
7.11.3 Click on “Zoom Next peak” to see if all lines are around in the peak center;
7.11.4 It’s also important to check:
In115: Check the In115 signal (good shape and the intensity is the same during the Tuning, if it has a signal loss some problem occurred);
7.11.5 Close the small window that contains the list of the elements detected;
7.11.6 Click on “Cancel” to close the window that allow us to change or add some peak;

7.12 In the mass Calibration window, click on “Continue” (Run the Thermo_LR_Narrow)

7.13 Click on: File → Open →Thermo_LR_Narrow

7.13.1 Click on “Manual calibration” (icon , and one small window with the elements will appear);

7.13.2 Click on: “Zoom Next peak” (icon);
7.13.3 Click on “Zoom Next peak” to see if all lines are around in the peak center;

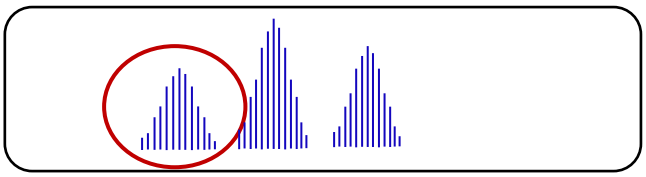
7.14 In the mass Calibration window, click on “Continue” (Run the Thermo_MR_Wide)

7.15 Click on: File → Open →Thermo_MR_Wide

7.15.1 Click on “Manual calibration” (icon , and one small window with the elements will appear);

7.15.2 Click on: “Zoom Next peak” (icon);
7.15.3 Click on “Zoom Next peak” to see if all lines are around in the peak center;
7.15.4 It’s important to check:

7.15.4.1 Si 28 (if not appear in the list: introduce the peak in the list)
7.15.4.1.1 Zoom the window around molar Mass [u] = 28;
7.15.4.1.2 We will see 3 peaks



7.15.4.1.3 Select the left one with double click;
7.15.4.1.4 Select Si28 in the small window and click on “Add”

7.15.5 In115: In = 10 % of sensibility comparing with LR value;
7.15.6 Check peak of Ga69
7.15.7 If everything it’s ok: save the calibration
*Normally the correction of Si28 will not appear, therefore, it’s necessary to save the calibration and reopen the window to see if the peak is in the correct position.

7.15.8 Close the small window with that contains the list with the elements detected;
7.15.8.1 Click on “Cancel”;

7.16 In the mass Calibration window, click on continue (Run the Thermo_MR_Narrow)

7.17 Click on: File → Open → Thermo _MR_Narrow

7.17.1 Click on “Manual calibration” (icon , and one small window with the elements will appear);

7.17.2 Click on: “Zoom Next peak” (icon);
7.17.3 Click on “Zoom Next peak” to see if all lines are around in the peak center;
7.17.4 Check: Si; Ga; In
7.17.5 In115 = 10 % of sensibility in LR
7.17.6 If everything it’s ok:
7.17.7 Close the small window with that contains the list with the elements detected;
7.17.7.1 Click in Cancel

7.18 In the mass Calibration window, click: on continue (to Run the Thermo_HR_Wide)

7.19 Click on: File → Open → Thermo_HR_Wide

7.19.1 Click on “Manual calibration” (icon , and one small window with the elements will appear);

7.19.2 Click on: “Zoom Next peak” (icon);
7.19.3 Click on “Zoom Next peak” to see if all lines are around in the peak center;
7.19.4 Check peak DIMER of Ar
7.19.4.1 $^{40}Ar+^{36}Ar$
7.19.4.2 $^{40}Ar+^{38}Ar$
7.19.4.3 $^{40}Ar+^{40}Ar$
7.19.4.4 ^{115}In 2% of LR signal
7.19.5 If everything it’s ok:
7.19.6 Close the small window with that contain the list that contains the list with the elements detected;
7.19.6.1 Click on “Cancel”;

7.20 In the mass Calibration window, click on “Continue” (Run the Thermo_HR_Narrow)

7.21 Click on: File → Open → Thermo_HR_Narrow;

7.21.1 Click on “Manual calibration” (icon , and one small window with the elements will appear);

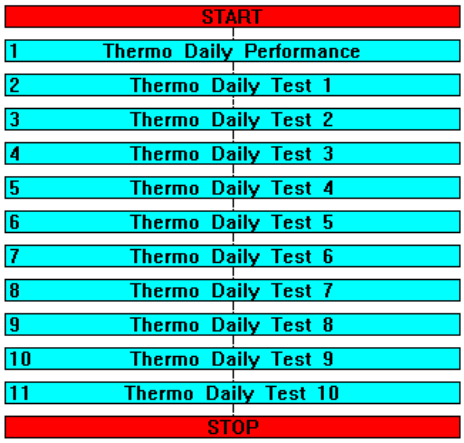
7.21.2 Click on: “Zoom Next peak” (icon);
7.21.3 Click on “Zoom Next peak” to see if all lines are around in the peak center;
7.21.4 Check peak DIMER of Ar
7.21.4.1 Ar 76
7.21.4.2 Ar 78
7.21.4.3 Ar 80
7.21.4.4 In 2% comparing with LR signal
7.21.5 If everything it’s ok:
7.21.6 Close the small window that contains the list with the elements detected;
7.21.6.1 Click on “Cancel”;

7.22 Close the window for Mass Calibration;
7.23 Click on “Cancel” in the Mass Calibration sequence window;
7.24 Close the Thermo Automasscal.seq.

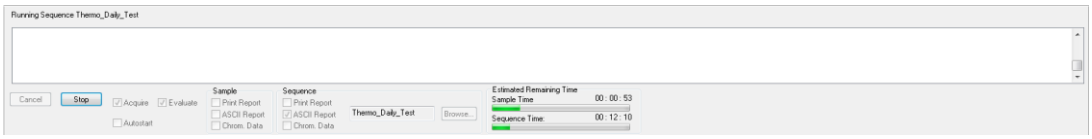
8. Daily test (to save the daily parameters)

8.1 Go to: File → Open → Daily sequences → Daily Test

A Daily test sequence will appear:



8.2 Reset item (icon)→ All
8.3 Go to: Actions → Able Auto lock mass;
8.4 Click on “Start”;
It will appear a window below the screen:



8.5 Select in the column Sequence
✓ ASCII Report
8.6 Browse: Thermo_Daily_Test.srcf
8.7 Click on: Run
8.8 In a small New window:
8.8.1 Select: Do not show this box again;
8.8.2 Continue;
8.8.3 Ok;
8.9 Wait for around 15 minutes;

After the Daily sequence finished:

8.10 Open the Excel software:
8.10.1 Ordinateur (My computer)

8.10.2 On the folder: Thermo Daily Test
8.10.3 Click on: Show → All the files
8.10.4 Click on the more recent file;
8.10.5 The file will be opened;
8.10.6 Click on: “Terminer” (Finish);
8.10.7 Press: “CTRL+A”;
8.10.8 Click on: “Convert to number”;
8.10.9 Check the parameters: RSD, R for MR and R for HR;
8.10.10 Save as (in the folder Daily test in Bureau) named: Daily test_ddmmyyyy;
8.10.11 Close the file.

9. Mass offset

9.1 Click on “Rune sequence” → Daily_sequences → Thermo_Determine_Mass Offset.seq;
9.2 Reset item (icon )→ All
9.3 Change Method: Ag_Emel_051216;
9.4 Click on “Start”;
9.5 Disable ASCII Report;
9.6 Insert the solution: Mass Off Set in the 5th position (for example);
9.7 Go to the auto sampler Window: click on 5th position →“Go to”
9.8 Click on: Run
9.9 Go to “View Result” (icon ) → Update Mass Offset → Save
9.10 After: click on “View Spectra” (icon ) and verify if the peaks are well positioned. Proceed with the sequence with Check Mass Offset.
9.11 Click on “Cancel”;
9.12 Close the sequence Mass off Set.

10. Create a sample sequence (new one)

10.1 Click on: File → Open
10.1.1 Elemental_conc_sequence.seq (It’s a default worksheet containing a ready sequence);
10.1.2 After open the sequence: Save as: search for File Rute Domingos (Search correct folder Claudia): Name: Ag_Claudia_DDMMYYYY.seq
10.1.3 Click on “Start”;
10.1.4 Change in the Analysis paramenter:
Method: Ag_Emel051216

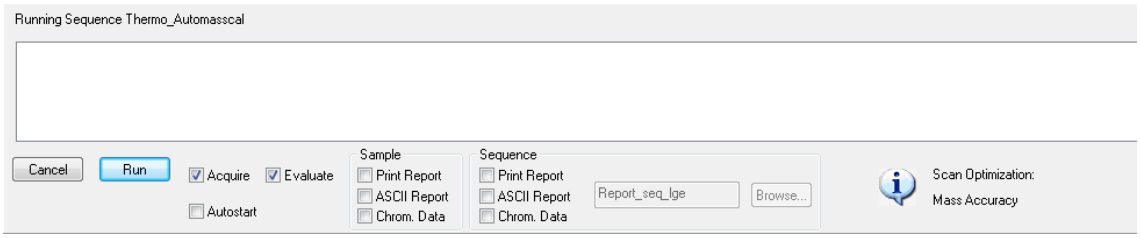
Tune Param: FAST (just to confirm)

Sampling: Nothing change

10.1.5 In the sequence analysis: to stop analysis after finished the sequence
PCL Stop: shutdown_23 Automatically

10.2 After check the correct sequence inputs, click on “Start” (icon) (One new window below the screen will appear);

10.3 Select in the window, the parameters:



10.4 Parameters

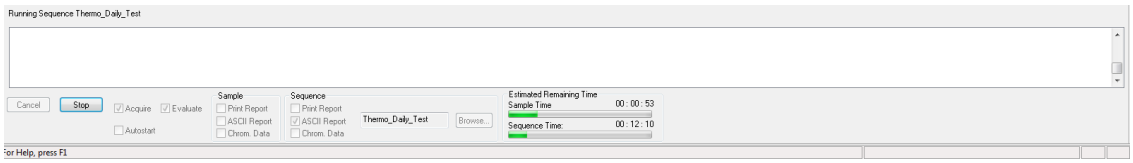
✓ Acquire
✓ Evaluate
✓ ASCII Report sample
✓ ASCII Report sequence

Browse: select “Report Sequence LGE.srcf

To initialize the sequence, click on “Run” (wait an automatic initialization);

10.5 Wait until the sequence is done;

Obs: It’s possible to have an estimative of the analyses seen the green box:



11. Acquire Data

To obtain the analyses results:

11.1 Go to → My compouter → Ordinateur →OS(C:) → Element → User → Root → data

11.2 Search the folder with the analyzes in the extension .ASC

11.3 Send by e-mail the results and NEVER insert a USB key in the computer.

12. Finish activities in the machine

After the analyses sequence had finished:

12.1 Save and Close the sequence;

12.2 Close the window Run Sequence;

12.3 Open the machine:

12.3.1 Unlock the auto sampler locker;

12.3.2 Open the big door sampler;

12.4 Close the gas valve ( ); ⬍

12.5 Close the Rinse 1 and Rinse 2 taps;

Cleaning Cones

Clean with Milli-Q water 18.2 MΩ and HNO_3 2% (v/v)

First cone: should be more careful, don't touch on the top of the cone, clean softly.

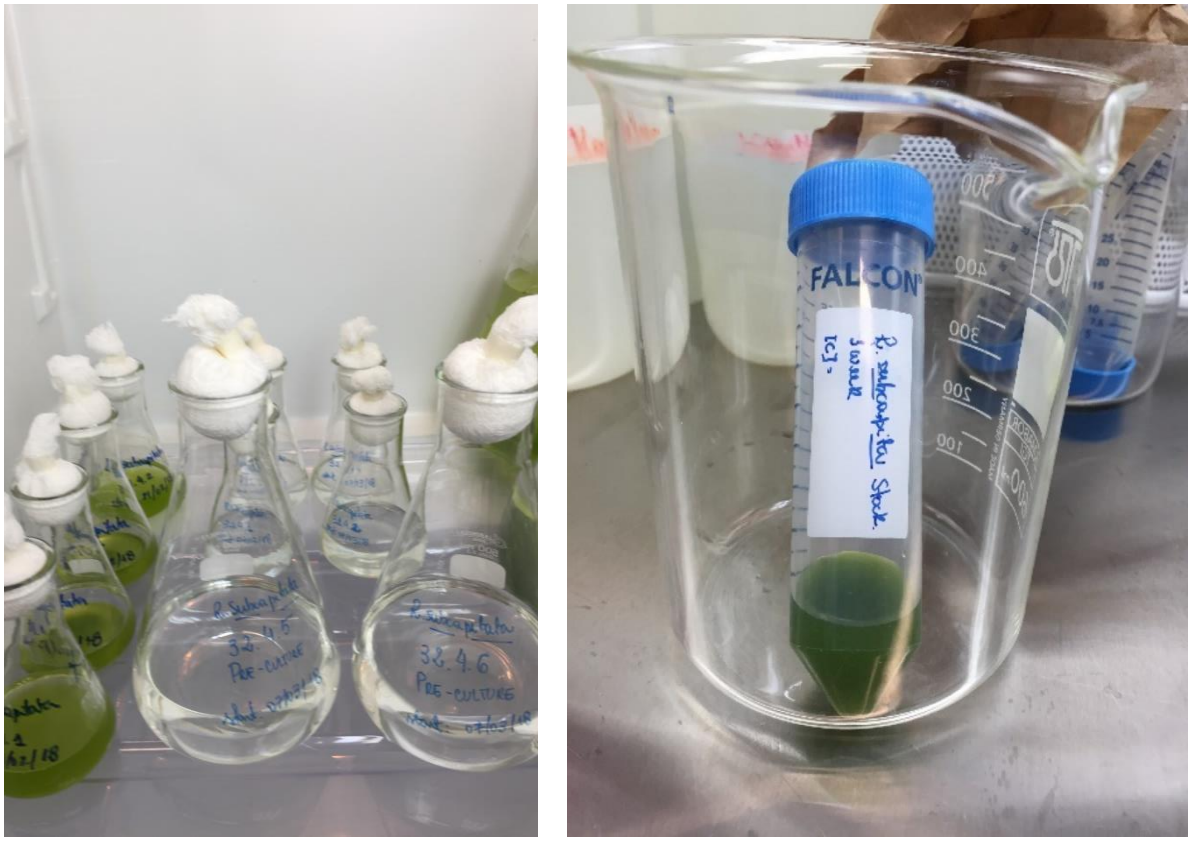
Second cone: don't need to be so careful but don't forget that the cone are sensibles.

Estimative time to Run a sequence in ICP-MS:

Switch: 30 min + 60 min (for wait)
Tuning: 30 min
Mass Calibration: 15 min
Daily Test: 15 min
Mass off Set: 5 min
Sequence: around 12-13 hours*
*Analysis of 120 samples only 1 element.

Appendix 4 - Pre culture preparation for R. subcapitata bioassays.

For pre culture preparation, under the same cultivation conditions as the organisms, a sufficient amount of solution was prepared (in this case, it was decided to use 2 flasks (Erlenmeyer with capacity for 500 mL) containing 300 mL Algae medium, starting from the initial concentration of seaweed 10^4 and 10^5 cells mL^{-1} to obtaining algal solution for further testing (Figura A4a). The day before the test, the cell number was counted to ensure the minimum initial number of cells required. On the day of the test, the algal inoculum was prepared by centrifuging the pre-culture solution (4,500 rpm, t = 15 minutes) and counting the number of cells.



(a) (b)

Figure A4 - Preculture (a) and inoculum (b) used for toxicity tests with R. subcapitata.

In properly decontaminated and previously sterilized conical flasks 100 mL of test solution prepared according to the established concentration and aseptic culture inoculum of known algal concentration were added.

Materials to be used properly decontaminated and sterilized assays.

Appendix 5 - Adaptation and acclimatation environment for Daphnia similis.

All generated acid residues were properly neutralized using commercial sodium hydroxide. Waste containing metals in concentrations above the maximum release limits determined by current legislation (CONAMA, 2005) was treated as contained in the UNESP waste manual, duly packaged, identified and forwarded to the UNESP Sorocaba Waste Commission, which is responsible for disposal of waste generated internally at the unit. Waste containing biological material (discarded organisms), as stated in the standard ABNT 12713 (2016), as the microcrustacean is not an autochthonous species and cannot be disposed of or thrown directly into the environment, were disposed of by prior exposure of the material to a solution of neutral detergent and sodium hypochlorite (left overnight). This practice was adopted in all situations in which organisms were discarded, in order to avoid the introduction of exotic species due to improper disposal. The same practice was adopted for proper disposal of algae.

In order to provide adequate cultivation conditions of the test organism during maintenance and testing, adaptations were made in the culture environment, as observed in Figure A5

a) b) c)

Figure A5 - Laboratory adaptations for the cultivation of D. similis. a) incubator with photoluminescence (diffuse lighting) and controlled temperature b) exclusive aquariums for aeration water reconstitution c) lighting box to aid in the maintenance and counting of the organism.

The last batch received was removed and transported directly from one laboratory to another to avoid thermal amplitude as a possible cause of non-adaptation of the culture. Transport was carried out using a small paper-filled Styrofoam box with the intention of preventing the vial which contained the organisms inside the box.

Initially, the cultures were maintained in two environments (Figure A6): controlled incubator with temperature (22 ± 1 ºC) and controlled illumination (<1000

lux) and photoperiod (16 h day and 8 dark hours) controlled (Figure A6a). The second location is the microbiology laboratory environment with constant room temperature (set at 22 °C) and natural light (Figure 11).

(a) (b)

Figure A6 - Acclimatation of the organism Daphnia similis a) in incubator and b) inside the laboratory.

Concomitantly, attempts were made to adapt the culture by varying the type of water used (Figure A7) initially using: Water 1 = reconstituted ultrapure water; Water 2 = cultivation water from source laboratory; Water 3 = algae medium with adjusted hardness. Subsequently, the addition of Selenium (2 µgL-1) was also tested based on the US EPA (2016) standard, eliminating cultivation with water from the original laboratory.

Figure A7 - Reconstituted *D. similis* cultivated water with (a) aeration and (b) containers containing the different cultivation waters of the test organism.

Regarding food, the preparation of the compound food was changed. Due to previous experiences with the high mortality rate when inserting compound feed containing filtered yeast into its formulation, an alternative procedure for the preparation of digested fish feed was adopted, according to ABNT (2016) standard.

Apparently a kind of water film formed that made it difficult to maintain the crop and soiled both the pipette and the new solution to which the organism would be exposed. To this end, 1 g of Tetramin ™ fish feed was added to 100 ml of ultrapure water and kept under stirring for 1 hour. After this period, the stirring is stopped and the solution is left for another 1 hour under decantation and then filtered using planktonic net as shown in Figura A8.

a) b) c)

Figure A8 - Fish feed a) digested, b) under filtration and c) filtered and ready for use.

Figure A9 shows the viability test result using different types of reconstituted water in the cultivation of *Daphnia similis*. This water type viability test result have tested 3 conditions:W1 = reconstituted ultrapure water; W2 = reconstituted Oligo LC medium; W3 = reconstituted ultrapure + Se 2 µg L-1 (USEPA 2002). Based on the reproduction chart of the organisms dispersed in the 3 different water compositions, it can be observed that the presence of Selenium provides greater reproduction and stability (smaller deviation) throughout the test. It can also be observed that the culture presents a good reproduction, allowing to perform toxicity assays with $AgNO_3$ and AgNPs.

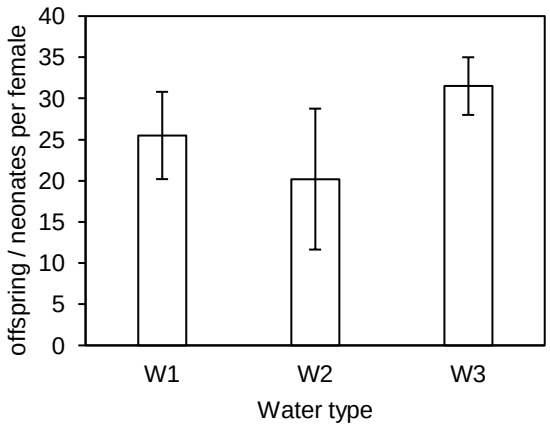


Figure A9 - Water type viability test result observing reproduction of Daphnia similis. W1 = reconstituted ultrapure water; W2 = reconstituted Oligo LC medium; W3 = reconstituted ultrapure + Se 2 µg L-1.

ABNT. Aquatic ecotoxicology - Acute toxicity - Test with Daphnia spp (Cladocera, Crustacea): 27 p. 2016.

CONAMA. RESOLUÇÃO No. 357. AMBIENTE, M. D. M.: 27 p. 2005.

USEPA, U. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Washington DC 2002.

Appendix 6 -General results of toxicity assays using *Daphnia similis.*

$AgNO_3$ – EC_{50}

Concentration	Survival / %	Offspring / neonate female^{-1}	SDOffspring	pHMean	pHSD	ODMean	ODSD	Conductivity	ConductivitySD
Control	90	17	3	7.58	0.02	-	-	-	-
1	90	17	3	7.55	0.01	-	-	-	-
2	100	9	3	7.62	0.00	-	-	-	-
5	70	4	1	7.52	0.01	-	-	-	-
10	10	0	0	7.63	0.00	-	-	-	-
25	0	0	0	7.66	0.00	-	-	-	-
50	0	0	0	-	-	-	-	-	-
100	0	0	0	-	-	-	-	-	-

AgCit – EC_{50}

Concentration	Immobility / %	pHMean	pHSD	ODMean	ODSD	Conductivity	ConductivitySD
0	0	7.74	0.02	5.63	0.23	187.65	0.85
1	0	7.60	0.07	4.45	0.23	187.15	4.23
5	10	7.72	0.01	4.25	0.18	188.80	0.50
10	10	7.73	0.01	4.08	0.14	189.50	0.75
50	100	7.59	0.21	3.73	0.11	189.63	0.04
100	100	7.71	0.01	3.68	0.13	191.58	1.83

AgPeg – EC_{50}

Concentration	Immobility / %	pHMean	pHSD	ODMean	ODSD	Conductivity	ConductivitySD
0	0	7.74	0.02	5.63	0.23	187.65	0.85
10	0	7.60	0.02	3.58	0.18	190.80	1.35
50	10	7.60	0.02	3.50	0.20	264.43	111.84
100	5	7.46	0.01	2.85	0.23	190.70	1.35
250	95	5.64	0.23	2.70	0.20	193.60	0.90
500	100	3.90	0.01	2.88	0.04	237.48	0.43

AgNP 1 μgL^{-1}

Concentration	Survival / %	Offspring / neonate female-1	SDOffspring	pHMean	pHSD	ODMean	ODSD	Conductivity	ConductivitySD
Control	97	79	11	7.56	0.02	5.82	0.52	-	-
AgCit1	80	99	6	7.45	0.17	5.43	0.03	-	-
AgCit1HS1	87	94	9	7.49	0.12	4.75	0.42	-	-
AgCit1HS2	97	80	11	7.47	0.08	4.80	0.37	-	-
AgPeg1	97	78	17	7.44	0.09	5.00	0.33	-	-
AgPeg1HS1	97	90	9	7.45	0.07	5.28	0.55	-	-
AgPeg1HS2	83	84	26	7.43	0.07	5.37	0.20	-	-

AgNP100 μgL^{-1}

Concentration	Survival / %	Offspring / neonate female-1	SDOffspring	pHMean	pHSD	ODMean	ODSD	Conductivity	ConductivitySD
Control	95	48	12	5.58	2.79	3.08	2.06	-	-
AgCit100	0	0	0	3.89	3.89	0.00	0.00	-	-
AgCit100HS1	0	3	0	5.05	3.37	4.45	4.45	-	-
AgCit100HS2	15	5	2	5.61	2.80	3.64	2.43	-	-
AgPeg100	50	31	18	5.48	2.74	3.41	2.28	-	-
AgPeg100HS1	30	33	2	5.38	2.69	4.80	3.20	-	-
AgPeg100HS2	70	45	15	5.31	2.65	3.26	2.17	-	-