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Universidade de Aveiro Departamento de Biologia
Ano 2015

Carla Sofia Leite Azevedo

Toxicidade de nanomateriais de ZnO decorados com Ag em *Daphnia magna*

Toxicity of Ag decorated ZnO nanomaterials to *Daphnia magna*

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Toxicity of Ag decorated ZnO nanomaterials to *Daphnia magna*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Investigadora auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro.

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palavras-chave Nanotecnologia, Ag, ZnO, misturas, *Daphnia magna*

resumo

A nanotecnologia é uma área em crescimento e os nanomateriais (NMs) podem ser encontrados numa vasta variedade de produtos como equipamentos ou dispositivos médicos e cosméticos. Os NMs atraem muita atenção devido à sua grande reatividade, resultado da sua elevada área de superfície em relação ao seu volume. Eles podem apresentar diferentes composições químicas, tamanhos e formas, o que pode influenciar o seu comportamento.

Devido ao aumento de produção e presença em bens de consumo, os NMs podem chegar ao ambiente devido a introdução direta ou indireta.

Apesar de muitos estudos se focarem na toxicidade dos NMs, diferentes resultados podem ser encontrados para NMs com a mesma composição química. Isto deve-se principalmente à influência de fatores abióticos e bióticos que podem alterar a biodisponibilidade dos NMs e por conseguinte a sua toxicidade, assim como à diversidade de características que estes materiais podem apresentar. A presença de outros NMs ou químicos no ambiente pode influenciar a sua toxicidade, aumentando-a ou diminuindo-a. Para além disto, têm sido desenvolvidos novos NMs formados por vários nanomateriais, aumentando as suas funcionalidades em comparação com o(a)s NMs/NPs isolado(a)s. Por estas razões é importante perceber como se irão comportar no ambiente.

Tendo isto em consideração, o principal objetivo deste trabalho foi avaliar a toxicidade de NMs compostos por ZnO-NM com Ag-NP na superfície (ZnO/Ag-NM) e tentar perceber se a toxicidade destes NMs pode ser prevista através da toxicidade individual dos seus componentes. Com este objetivo a toxicidade individual e em mistura de ZnO-NM e de Ag-NP foi avaliada no organismo *Daphnia magna* e posteriormente comparada com a toxicidade de ZnO/Ag-NM. Para isso, foram realizados testes de imobilização e reprodução. Para avaliar a toxicidade da mistura e dos ZnO/Ag-NM também foi utilizada a ferramenta informática MixTox, baseada no modelo de adição de concentração e foram explorados possíveis desvios como sinergismo/antagonismo (S/A), desvio dependente da dose (DL) e desvio dependente do ratio químico (DR).

Os ZnO-NM e as Ag-NPs demonstraram um esperado aumento dose-resposta para *Daphnia magna*. Foi verificada, para ambos os NMs, uma diminuição da sobrevivência ao fim de 48h e uma diminuição do número de neonatos produzido durante 21 dias. As Ag-NPs foram as que demonstraram maior toxicidade.

A mistura apresentou um desvio dependente da dose (DL) para a imobilização e para a reprodução foi observado sinergismo.

Os ZnO/Ag-NMs apresentaram maior toxicidade do que os ZnO-NM individualmente. Quando os resultados foram analisados com o MixTox foi observado um desvio dependente do químico (DR) para a imobilização e um desvio dependente das doses usadas (DL) para a reprodução.

Este estudo demonstra que tanto a mistura efetuada em laboratório como a previsão baseada nos resultados de toxicidade dos ZnO/Ag-NM não serão baseados no mesmo comportamento dos seus componentes e demonstra também a importância de ter em consideração a interação NM-NM aquando da avaliação da toxicidade dos NMs.

keywords Nanotechnology, Ag, ZnO, mixture, *Daphnia magna*

abstract

Nanotechnology is a rising field and nanomaterials (NMs) can now be found in a vast variety of products that can go from medical equipment to cosmetics. NMs attract much attention due to their high reactivity, a result of high surface area to volume ratio. They can present different chemical compositions, sizes and shapes which can alter their behaviour.

Due to their increase of production and presence in consumer products, NMs can end up in the environment due to unintentional or intentional release.

Although many studies have focused on the toxicity of NMs, different results can be found for NMs with the same chemical composition. This is due to the fact that abiotic and biotic factors can alter the NMs bioavailability and therefore their toxicity, along with the diversity of their inherent characteristics. The presence of other NMs or chemicals in the environment can also affect NMs toxicity, increasing or decreasing their toxicity. Also, new nanomaterials combining NM-NM are being development due to their enhancing characteristics when compared to NMs or nanoparticles (NPs) alone. Therefore, it is important to understand how they will behave in the environment.

Taking this into account, the main objective of this work was to evaluate the toxicity of a NM composed by ZnO-NM with Ag-NP on its surface (ZnO/Ag-NM) and try to understand if its toxicity can be predicted by the toxicity of the single components. With this purpose, the toxicity in *Daphnia magna* was evaluated to ZnO-NM and Ag-NP as single components, as a laboratory mixture and then compared to the toxicity of the ZnO/Ag-NM. To assess toxicity, immobilization and reproduction tests were performed. Also, the mixture toxicity and the toxicity of the ZnO/Ag-NM were analysed using the MixTox tool, based on the concentration addition model and possible deviations for synergism/antagonism (S/A), dose-level (DL) and dose-ratio (DR) were explored.

ZnO-NM and Ag-NPs showed an increase toxicity to *Daphnia magna* with increasing concentrations. Decrease of survival after 48h and decrease in the number of neonates produce during 21 days were observed for both NMs with Ag-NPs demonstrating the highest toxicity.

The mixture exposures showed a deviation dependency on the doses used (DL) for immobilisation and for reproduction a synergism deviation was observed.

ZnO/Ag-NM showed higher toxicity when comparing to the ZnO-NM alone. When analysing the results with the MixTox tool a deviation dependent on the chemical present (DR) was observed for immobilization and a dose level deviation (DL) for reproduction.

This study demonstrates that both, the mixture and the ZnO/Ag-NM, will not behave as their components and the toxicity cannot be predicted by them, highlighting the importance of taking into account the interaction NM-NM when assessing NMs toxicity.

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Chapter I

General Introduction

1. General Introduction

1.1. Nanotechnology

According to the European Commission a nanomaterial (NM) is 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'' (European Commission 2010). Although nanomaterials always exist in nature, engineered NMs had a great increase in production during the XXI century, making nanotechnology one of the fastest growing technologies in the world (Piccinno et al. 2012). Due to their high surface area to volume ratio, which make them more reactive, NMs present unique characteristics comparing to their bulk material (Bondarenko et al. 2013; Lowry et al. 2012). These characteristics make them very appealing for the application in a huge variety of products that go from personal care products to medical equipment (Al-Mubaddel et al. 2012).

1.2. Types of nanomaterials and their applications

Nanomaterials can be divided in natural nanomaterials and anthropogenic nanomaterials. Natural nanomaterials can be formed due to geological processes (e.g. volcanic activity) or biological processes (e.g. degradation of biological matter) (Handy et al. 2008) and they have always been present in the environment. Anthropogenic nanomaterials include unintentionally produced nanomaterials (e.g. emissions from motor vehicles) and engineered nanomaterials (ENMs) (Kumar et al. 2014).

ENMs can be manufactured with the chemical composition, size and shape desired which allows their implementation in different fields such as medical products, pharmaceuticals, electronics, personal care products and much more (Nowack & Bucheli 2007; Elder et al. 2007).

According to the number of dimensions in the nanoscale, NMs can be identified as nanoparticles, nanofibers and nanoplates. A nanoparticle has the three dimensions in the nanoscale range, while the nanofiber has two dimensions in the nanoscale range being the third dimension significantly larger; a nanoplate has one dimension in the nanoscale range being the other two dimensions significantly larger (ISO/TS 12901-1:2012).

Besides their dimension, NMs can also be divided by their chemical composition which include five classes:

- *Carbon nanomaterials*

Carbon nanomaterials, such as fullerenes and nanotubes, have a vast range of applications that can go from plastics, components in electronics to water purification systems (Bhatt & Tripathi 2010).

- *Metal oxide nanomaterials*

Titanium dioxide (TiO_2), zinc oxide (ZnO) and cerium dioxide (CeO_2) are some examples of metal oxide nanomaterials. CeO_2 can improve emission quality when used in diesel fuels and can also be used in gas sensors and oxygen pumps (Klaine et al. 2008). ZnO and TiO_2 have the capacity to attenuate ultraviolet radiation and can be found in sunscreens and skin care products (Lee & An 2013).

- *Semi-conductor materials*

Semi-conductor materials include quantum dots (QDs) which have mostly medical applications such as medical imaging and targeted therapeutics (Klaine et al. 2008). They are composed by a metal or a semi-conductor core such as cadmium selenide (CdSe) or zinc selenide (ZnSe) and a shell that can be made of silica or zinc sulfide (ZnS) to protect from oxidation processes (Bhatt & Tripathi 2010).

- *Zero-valent metals*

Zero-valent metals include silver (Ag), iron (Fe) and gold (Au). They can be used to remove nitrates from water, soil and sediments during environmental remediation (Bhatt & Tripathi 2010). Due to their antimicrobial proprieties Ag-NMs can also be found in textiles, cosmetics and as a coating of medical devices (Chernousova & Epple 2013).

- *Nanopolymers*

Nanopolymers are multifunctional polymers whose physical proprieties can be controlled. They can be applied in nanolatex, coloured glasses, drug delivery and chemical sensors, etc (Klaine et al. 2008).

Figure 1 shows the estimate annual global production of 10 ENMS, where silicon dioxide (SiO_2), TiO_2, ZnO and carbon nanotubes can be highlighted as the most produced NMs (Bondarenko et al. 2013).

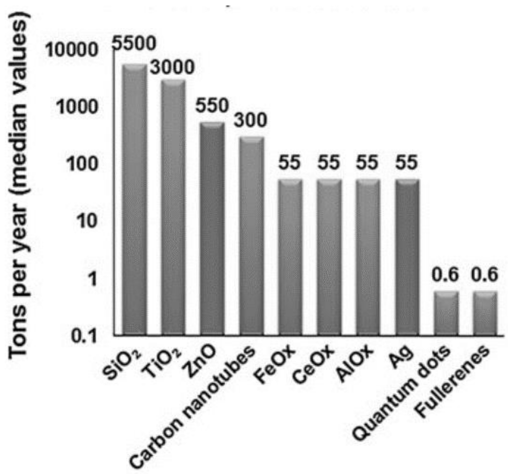

Figure 1. Estimate annual global production of ENMs. (Adapted from Bondarenko et al. 2013)

With the purpose of inhibiting overgrowth during synthesis and decreasing aggregation of NMs, several capping agents can be added to the surface of NMs. Some examples of capping agents are polymers, hydrocarbons, polycarboxylic acids and cationic surfactant (Tolaymat et al. 2010; Niu & Li 2014).

To enhance their characteristics and efficiency, NMs are currently being combined with other nanomaterials. For instance, ZnO-NMs can be added to the surface of carbon nanotubes in order to enhance electron transition, which can play an important role in electronics (Li et al. 2015). Fe_3O_4 nanoparticles decorating TiO_2 nanofibers can also be used to improve battery life and performance (Wang et al. 2015). In the literature, many nanomaterials have been combined with metals with the ultimate goal of improving the photocatalytic activity. Some examples are Ag/TiO_2 (Geetha et al. 2015), CuO/ZnO (Mageshwari et al. 2015), ZnO/Ag/Cu (Shvalagin et al. 2004) and mainly combinations of silver and zinc oxide nanomaterials (Yin et al. 2012; Georgekutty et al. 2008;

Ren et al. 2010a; Wang et al. 2012; Wu et al. 2013). Metals like ZnO and TiO_2 are known for their photocatalysis proprieties which are promoted upon illumination with UV radiation (Ullah & Dutta 2008). The addition of metals like Ag to the surface of TiO_2 and ZnO increase the absorption spectrum which makes photocatalysis possible with natural light (Rekha et al. 2010). Since photocatalysis can be used as a process of decomposition of organic and inorganic contaminants and bacteria disinfection (Ullah & Dutta 2008) these kind of improvement can be very important to environmental remediation.

1.3. Nanomaterials in the environment: emissions and abiotic and biotic fate

Emissions

ENMs have been produced at higher scales and they are present in a widely variety of products, which can, ultimately, make them end up in the environment due to unintentional and intentional release. Unintentional release can occur during the entire life cycle of NMS during manufacturing, direct release from products and at the end of their life-cycle, due to incorrect disposal and treatment of residues (Savolainen et al. 2015; Nowack & Bucheli 2007).

For example Ag-NP present in textiles can be release to air and water during wearing and washing (Wigger et al. 2015). Wigger et al. (2015) showed that after fourteen and seventeen washing cycles, the total amount of Ag-NPs, present in cotton and polyester textiles, respectively, was released to the aquatic environment. The results also demonstrated that Ag-NPs present in the fabric can as well be release to air, especially in cotton textiles. The application of TiO_2-NM and ZnO-NM in sunscreens leads to a release of these nanomaterials to the aquatic environment during bathing or swimming (Wiench et al. 2009). When used in external paints, TiO_2-NM can be detached by natural weather conditions and transported to soils and then to the aquatic ecosystems through runoff (Kaegi et al. 2008).

NMs can be intentionally released to the environment during their application into environmental remediation (Nowack & Bucheli 2007; Geetha et al. 2015). Depending on the NMs applied, different processes can be used in water and soil treatments. TiO_2-NM can degrade organic matter due to photocatalysis or NMs such as Fe_3O_4 can absorb arsenic and/or chromium, which will removed from media using magnetic or gravitational fields (Sánchez et al. 2011).

Abiotic fate

The behavior of NMs, and therefore their toxicity, depend on NMs characteristics and environment factors. Their small size, chemical composition, dissolution rate and agglomeration/aggregation processes are some examples of NMs characteristics which can influence their toxicity. Meanwhile, the environment factors such as the pH, ionic strength, organic matter and water hardness also play a crucial role in their behavior (Handy et al. 2008).

Once into the aquatic environment, NMs can suffer different processes which can alter their toxicity (figure 2). They can form not only agglomerates (weak bond, reversal) or aggregates (strong bond, irreversible) with themselves but also through heteroaggregation bond to other compounds present in the environment, like organic matter (OM), clay or silicates (Lowry et al. 2012). Aggregation can alter NMs toxicity by decreasing their surface area and therefore their reactivity (Lowry et al. 2012). The decrease in the surface area can subsequently decrease the dissolution rates increasing the persistence of NMs in the environment. The NMs accumulated in the sediment can suffer resuspension over time due to turbulence of the water (Markus et al. 2015).

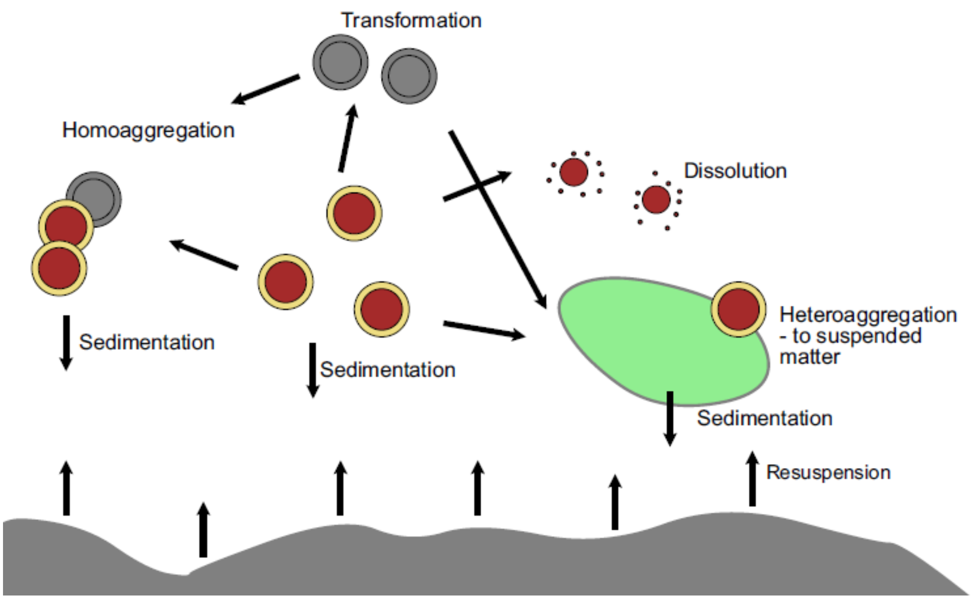

Figure 2: Different processes that nanomaterials can suffer in the aquatic environment (Markus et al. 2015)

Zhou at al. (2010) showed that ZnO-NM sedimentation is highly related to the concentration of organic matter (OM) and the ionic strength (IS) of the media. Sedimentation rates in freshwater (low IS, high OM) and in seawater (low OM, high IS) were analyzed for ZnO-NM and high sedimentation rates in seawater are expected whereas in freshwater ZnO-NMs were more stable during the 6h experiment period (Zhou & Keller 2010).

Besides increasing the stabilization of NMs, OM can also promote redispersion of agglomerates (Loosli et al. 2015). Redispersion can also occur during light exposure and temperature variation (Loosli et al. 2015). The pH can also influence the degree of aggregation since variation of pH can alter the surface charge of the NMs. Higher differences between the environmental pH and the NMs point-of-zero-charge will lead to higher stability (Garner & Keller 2014).

The aggregation and sedimentation of NMs will influence their potential transport and therefore their fate, bioavailability and toxicity (Garner & Keller 2014). Studies show that NMs are, usually, rapidly removed from the water column being partitioned between the sediment and the organisms (Bour et al. 2015). NMs which stay dispersed in the water are more available to pelagic organisms while NMs that accumulate in the sediment (lower bioavailability in the water) become more available to benthic organisms (Garner & Keller 2014).

NMs with high dissolution rates tend to be available to organisms during a short period of time due to rapid degradation while NMs with low dissolution rates tend to have a high persistence in the environment. Zn, Ag, aluminum (Al) and copper (Cu) nanomaterials have high rates of dissolution when compared with ceria, TiO_2 and carbon-based nanomaterials. The first ones dissolve over the course of days while the last ones do not show any dissolution over months (Garner et al. 2015).

NMs can form heteroaggregates with inorganic materials present in the aquatic environment such as clay and silicates (Loosli et al. 2015). They can also suffer transformations like sulfidation and oxidation (Dwivedi et al. 2015; Garner & Keller 2014). Oxidation of NMs can lead to the formation of other compounds such as AgCl (reaction of Ag^+ with Cl^-) and $CuCO_3$ (reaction of Cu^+ with CO_3^{2-}). Sulfidation occurs mainly in anaerobic conditions by the substitution of ions with sulfide. Some examples are the formation of ZnS, CdS and Ag_2S (Garner & Keller 2014). All these compounds are considered less bioavailable and therefore prone to exert less toxicity.

Biotic fate

When in the aquatic environment NMs can attach to the surface of organisms but they can also enter the organisms through the skin, the gills or the digestive tract (Garner & Keller 2014). Organisms can be exposed to NMs due to contaminated water or sediment (bioconcentration), due to contaminated food or from both sources (bioaccumulation) (Bour et al. 2015). Figure 3 shows the different routes of exposure for aquatic organisms emphasizing the exposure through the water phase and/or through the food (Baun, Hartmann, et al. 2008). If NMs are accumulated in/by the organisms, biomagnification in the trophic chain might be observed, where the contaminant concentration in organisms is increasing with their position in the trophic chain (Bour et al. 2015).

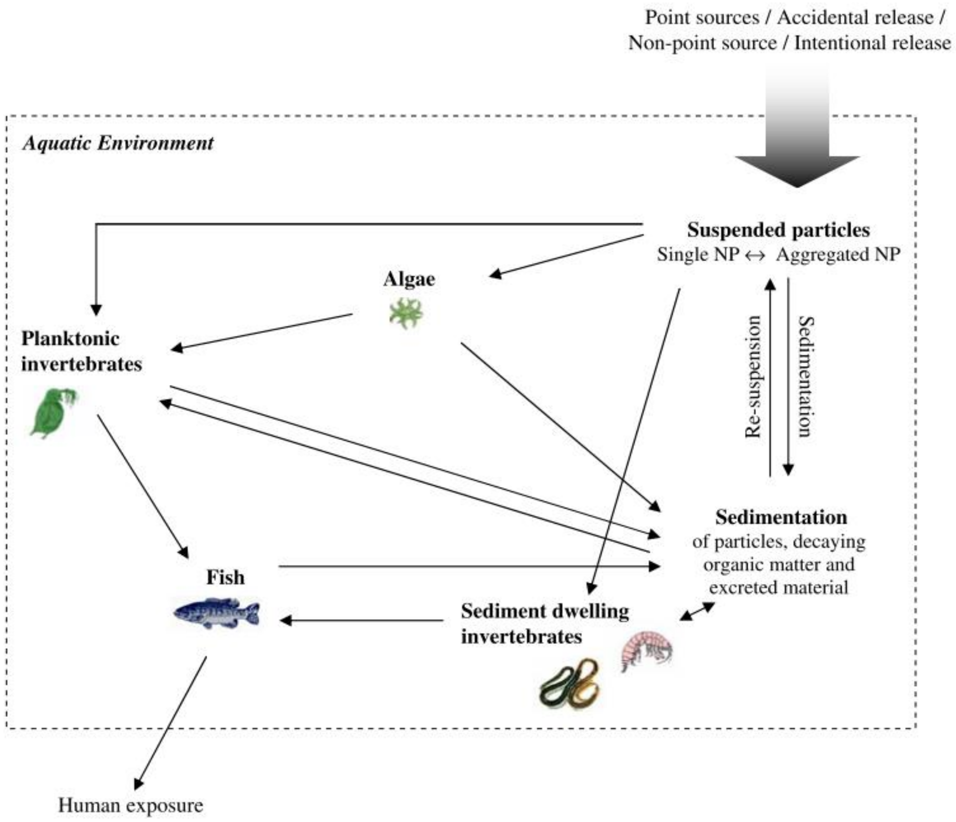

Figure 3. Exposures routes of NMs in an aquatic environment (Baun et al. (2008))

A study from Cleveland et al. (2012) held in an estuarine mesocosm with seawater contaminated with Ag-NP showed that these NPs accumulated in plants, shrimp, clams and snails being the accumulation higher in the last two. Exposure of the amphipod *Leptocheirus plumulosus* (Jackson et al. 2012) and the cladoceran *Daphnia magna* (Fouqueray et al. 2012) to NMs through contaminated food (algae) showed negative effects, such as high mortality as well as impairment of reproduction, showing the importance of accumulation through trophic chain. Zhao et al. (2010) also showed that more than 70% of Ag-NPs accumulated in *Daphnia magna* was also through the dietary route. But, looking at the work of Ribeiro et al. (2015), in this particular case, Ag transfer may not be in the nanoparticulate form as algae are not able to internalize Ag-NP but its counterpart ions or complexes.

1.4. Nanomaterials toxicity

Engineered nanomaterials have enormous advantages in terms of applicability and improvement of products but they can also bring disadvantages to the ecosystems and to humans. Some reviews can be found in the literature regarding the toxicity of NMs and their harmful effects (Bondarenko et al. 2013; Fabrega et al. 2011; Zhao & Liu 2012; Ma et al. 2013; Adam et al. 2015; Tourinho et al. 2015).

Bondarenko et al. (2013) collected data from the literature regarding the toxicity of Ag-NP, CuO-NP, ZnO-NP and their respectively soluble metal salts. The results collected focus on bacteria, crustaceans, algae, fish, nematodes, yeasts, protozoa and mammalian cell lines. From these eight organism/cells crustaceans were the more sensitive to both NPs and respective salts. Ag-NP and the Ag salt showed higher toxicity level for all organisms/cells and ZnO-NP was the one with most similarity between the toxicity of the NP and the toxicity of the salt, showing that the toxicity of ZnO-NP can be related to the dissolved ions.

Adam et al. (2015) produced aquatic species sensitivity distributions (SSDs) for ZnO and CuO nanoparticles and their bulk materials. They collected data for bacteria, algae, protozoa, yeasts, nematoda, crustacea and fish. The SSDs showed the sensitivity variation of different species to a chemical and allows the calculation of a hazard concentration 5% (HC_5). The HC_5 reflects the lowest concentration in which 95% of the species will be protected (Adam et al. 2015). The freshwater algae was the most sensitive to both ZnO and bulk. The HC_5 for both was 0.06 $mg.Zn.L^{-1}$ showing the similarity between the toxicity of ions and NPs, this similarity was also demonstrated by Bondarenko et al. (2013) and by Lopes et al. (2014). For CuO, the HC_5 was very different between the NPs and the bulk, being 0.15 and 6.19 $mg.Cu.L^{-1}$, respectively. This shows that although the toxicity of some NMs might be related to the dissolution of ions, this is not a rule for every NM.

In a recent study by Garner et al. (2015), SSDs for freshwater species were also build gathering data for Ag-NM, Ag-NM coated with PVP, C_{60}-NM, CuO-NM, Cu-NM, TiO_2-NM, ZnO-NM, CeO_2-NM, CNTs and Al_2O_3. When comparing HC_5 values the NMS presenting the lowest values were Ag coated with PVP, followed by Ag, Cu, CuO, ZnO, C_{60}, CeO_2, TiO_2, CNTs and Al_2O_3, by increasing order.

The results from the studies mentioned above demonstrated the importance of testing the effects of NMs in aquatic species, since these ones showed the high sensibilities. This is very important because the aquatic environment is usually the final destination of NMs. Moreover, based on the studies mentioned above Ag seems to be the NM that can cause higher toxicity levels to organisms.

1.5. Mixture toxicity

Although most of the toxicity tests found on the literature focus on the toxicity of one single nanomaterial, in the environment organisms may be exposed not only to one NM but to a combination of different NMs and other natural and unnatural stressors such as pesticides, PAHs and abiotic factors. These combined exposures may alter the toxicity of NMs when compared to the effects of the single NM exposures.

In order to analyze the joint toxicity of chemicals two reference models based on the mode of action (MoA) of the single chemicals can be applied: concentration addition (CA) and independent action (IA). These two models assume that the components of the mixture do not interact with each other meaning that they do not interfere with the biological action of each other. If the MoA of a chemical is not known or it is ambiguous, both the CA and IA model are applied (Loureiro et al. 2010).

The CA model is based on the premise that the chemicals have the same MoA and it was first formulated by pharmacologists in 1926 (Loewe & Muischnek 1926). This conceptual model is defined by the following equation:

$$\sum_{\mathrm{i}=1}^{\mathrm{n}} \frac{\mathrm{C}i}{\mathrm{ECx}_{i}} = 1$$

where C*i* is the concentration of the chemical *i* in the mixture, and ECx_i is the effect concentration of the chemical *i* that causes the same effect as the mixture does. The dimensional toxic unit (TU) can also be assessed by the quotient Ci/ECx_i, which quantifies the relative contribution to toxicity of the individual chemical *i* in the mixture of *n* chemicals (Jonker et al. 2005). The relative contribution of the individual chemical is important since most chemicals have different toxicities in a mixture and a small amount of a very toxic chemical in a mixture can cause a superior effect than a higher amount of a less toxic chemical (Jonker et al. 2005). Usually the ECx applied corresponds to the EC50 because it is the effect concentration less susceptible to variability (Jonker et al. 2005).

The IA model is based on the assumption that the chemicals have a different MoA. Mathematically, the IA model is expressed as:

$$Y = \mu \max \prod_{i=1}^{n} qi(Ci)$$

where Y is the biological response, C*i* the concentration of chemical *i* in the mixture; *qi(Ci)* is the probability of non-response, μmax is the control response for endpoint, Π is the multiplication function (Jonker et al. 2005). In this conceptual model, only components that cause an effect are considered. Components at concentrations below the threshold effect will not contribute to the toxicity of the mixture, meaning that if this condition is observed for all components there will be no combination effect (van Gestel et al. 2006).

If the chemicals affect each other, such as in terms of bioavailability, MoA and behaviour after uptake, deviations from the reference models can be observed (Loureiro et al. 2010). These deviations can be of synergism/antagonism, dose level deviation and dose ratio deviation. Synergism is observed when the mixture has a higher toxicity than the one expected and antagonism when the toxicity is lower. A dose level (DL) deviation can be observed when the toxicity of the mixture differ at high and low dose levels and a dose ratio (DR) deviation can be observed when the toxicity of the mixture is dependent of its composition (e.g. which chemical is mainly responsible for inducing the toxicity of the mixture) (Jonker et al. 2005).

Several studies can be found in the literature successfully applying the CA and IA models for binary mixtures of chemicals (Loureiro et al. 2010; Loureiro et al. 2009; Pitombeira de Figuerêdo et al. 2015; Silva et al. 2015) or for mixtures of a chemical and an abiotic factor (Azevedo et al. 2015; Ferreira et al. 2010; Lima et al. 2011;Ribeiro et al. 2011).

A recent EFSA report (European Food Safety Authority 2015) advices the use of the CA model to analyze the toxicity of chemical mixtures since it is considered the most conservative conceptual model. The report also highlights that when dealing with environmental mixtures the mechanistic pathway should be the main focus instead of the specific mode of action.

Although some studies assessed the combined effects of NMs with other compounds, very few studies can be found regarding the mixture toxicity of NM-NM. Concerning the mixture toxicity of NMs and other compounds, the joint effects of some NMs (e.g. CeO_2, CNT) and phenanthrene can be found in the literature (Baun et al. 2008; Cui et al. 2011; Tourinho et al. 2015). Tourinho et al. (2014) showed that the presence of CeO_2 did not affect the toxicity of phenanthrene in the isopod *Porcellionides pruinosus* and in the springtail *Folsomia candida*. Some studies with TiO_2 present in a mixture can also be found. TiO_2-NM can increase the bioaccumulation of copper and cadmium in *Daphnia magna* (Fan et al. 2011) and in *Cyprinus carpio* (Zhang et al. 2007), respectively. Moreover, combined exposures to TiO_2-NM and bisphenol revealed a synergistic effect to zebrafish embryos (Yan et al. 2014).

Regarding the toxicity of a mixture composed by only NMs, to our knowledge there are only two studies in the literature. Dimkpa et al. (2015) observed that the effects of CuO-NM, on the shoot and root growth of *Phaseolus vulgaris*, were higher when compared to the single exposures of CuO-NM and ZnO-NM. Also, Zhao et al. (2012) evaluated the effects of a mixture exposure with CuO-NM and ZnO-NM to *Daphnia magna*. The mixture showed a synergistic effect on survival and reproduction when compared to the NMs alone.

With the increasing production of NMs and the emerging of new NMs combining NM-NM that can ultimately end up in the environment, it is important to understand how the presence of more than one NM in a mixture can influence their toxicity to the organisms.

1.6. Aims

New emerging nanomaterials are prone to induce hazard to aquatic organisms if released into the aquatic environment. Considering the new emerging NMs composed by different NMs and NPs, the aim of this work was to understand whether the toxicity of a composed NM, in this case ZnO-NM with Ag-NP on its surface (ZnO/Ag-NM), can be predicted through the individual toxicity of the components. To achieve this, firstly the effects of ZnO-NM and Ag-NP were singly evaluated in the model organism *Daphnia magna* and then in a binary mixture. These results were then compared with the toxicity of the ZnO/Ag-NM. The parameters chosen to evaluate the toxicity were the survival and the reproduction.

This thesis is divided in three chapters: I) the current one where an introduction and state of the art regarding NMs in the environment are carried out, followed by the work objectives; II) the main study of this thesis, where the scientific paper "Toxicity of Ag decorated ZnO nanomaterials to *Daphnia magna*" is presented and originated from a multidisciplinary collaboration; and III) the general discussion and conclusions are presented in a summarized way.

1.7. References

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Chapter II

Toxicity of Ag decorated ZnO nanomaterials to *Daphnia magna*

2. Toxicity of Ag decorated ZnO nanomaterials to *Daphnia magna*

2.1. Introduction

Nanotechnology is a fasting growing industry and nanomaterials can nowadays be found in a vast variety of products such as cosmetics, solar panels, antibacterial products and electronics (Piccinno et al. 2012). In the last decade the production of nanomaterials had an exponential increase and now new research is focusing on increasing their performance and applicability.

Some nanomaterials such as zinc oxide (ZnO) and titanium dioxide (TiO_2) present high photocatalytic activity. Photocatalysis has many applications such as in ultraviolet (UV) absorbing material, white pigment that gives color to paper and paint, antibacterial and in water and soil remediation (Ullah & Dutta 2008; Yunus et al. 2012). In this last one photocatalysis is responsible for the oxidation of organic pollutants into nontoxic materials (Yunus et al. 2012). With the purpose of increasing the photocatalytic proprieties, zinc oxide nanomaterials (ZnO-NM) are now being combined with silver nanomaterials (Ag-NM) (Zheng et al. 2008; Georgekutty et al. 2008). This combination will allow the photocatalysis to occur not only with UV radiation but also with natural light (Rekha et al. 2010).

Several toxicity studies had demonstrated the negative effects of ZnO and Ag nanomaterials to aquatic organisms as can be found in the reviews of Ma et al. (2013) and of Fabrega et al. (2011), respectively. Although the toxicity of the individual nanomaterials is known, the toxicity as a combined component is not understood. Taking this into account, it is important to understand if these combinations of nanomaterials will behave as its components, being the toxicity predicted based on the individual components, or if a different toxicity and interaction with the biotic compartment will be observed.

Having this into consideration, the aim of this work was to understand if by having the individual toxicity of the nanomaterials we can predict the toxicity of the components together. This will be extremely important regarding regulatory issues, as NMs are included in the REACH regulation, meeting the regulations' substance definition. In addition, the recommendation allows also their inclusion in other European regulations, like the CLP which deals directly with mixtures. For that the toxicity of a nanomaterial, composed by ZnO with Ag-NP on its surface, was assessed, along with the toxicity assessment of ZnO-NM and Ag-NP alone and combined. To evaluate the toxicity, immobilization and reproduction tests were performed with *Daphnia magna* and values of LC_{50} and EC_{50} were calculated from the single exposures, and used to set up the mixture toxicity experimental design.

2.2. *Material and Methods*

Nanomaterials

The test substances were provided by the Department of Physics of the University of Aveiro. Zinc oxide with a tetrapod shape (ZnO-NM), silver nanoparticles (Ag-NP) and zinc oxide tetrapods decorated with silver nanoparticles (ZnO/Ag-NM) were provided as powders. ZnO/Ag-NM presented 1% of Ag-NP on the surface of the ZnO tetrapods.

All stock solutions (5 $mg.L^{-1}$) were prepared in ASTM moderated hard water (ASTM 1980) with sonication during 15 minutes. The stock solutions were sampled for characterization at time zero and after 48h. The ZnO-NM and ZnO/Ag-NM stock solutions were analyzed through Scanning electron microscope (SEM) and the stock solution for Ag-NPs through Scanning transmission electron microscopy (STEM).

Test Organisms

All the experiments were conducted with *Daphnia magna* (clone Beak) as test organisms. Cultures were maintained in 1L glass jars with ASTM and medium was changed every other day. Organisms were fed with *Raphidocelis subcapitata* at a concentration of $3x10^5$ $cell.mL^{-1}$ plus a seaweed extract (6 $ml.L^{-1}$) as supplement. Cultures were kept at 20±1ºC and under a 16:8h light:dark photoperiod. Neonates from the third and fourth brood were used to perform the toxicity tests.

Immobilization tests

Immobilization tests were perform based on the OECD guideline 202 (OECD 2004).

Tests were performed with five replicates per concentration plus the control and five neonates with less than 24h were transferred to 50ml glass beakers (replicate). Tests were maintained at room temperature of 20±1ºC and a 16:8h light:dark photoperiod and daphnids were not fed during the entire experiment. After 24h and 48h, immobilization (inability to swim after gentle agitation of the beaker) and mortality were assessed. The concentration that caused the immobilization of 50% of the neonates (LC_{50}) was calculated (see below for details).

For ZnO-NM treatments the concentrations ranged between 0.5 to 1.3 mg.Zn.L^{-1}, for Ag-NP between 0.05 to 0.25 mg.Ag.L^{-1} and for ZnO/Ag-NM the concentrations range between 0.13 to 0.63 mg.ZnO/Ag.L^{-1} (1% Ag).

Reproduction tests

Reproduction tests were based on the OECD guideline 211 (OECD 1998).

For each concentration plus the control 10 replicates with one neonate were used. Neonates with less than 24h were transferred to 50ml glass beakers and maintained in culture conditions during 21 days. The medium was renewed every two days and the daphnids fed every day with the algae *R. subcapitata*. The number of offspring of each brood were counted and removed from the beakers and the mortality of offspring and parental daphnids were also recorded.

Daphnids were measured in the beginning and in the end of the test to assess differences in size between the treatments and the control. Dissolved oxygen, conductivity and pH were measured at the beginning, middle and end of the test in the old and new medium.

For ZnO-NM treatments the concentrations ranged between 0.1 to 0.4 mg.Zn.L^{-1}, for Ag between 0.095 to 0.5 mg.Ag.L^{-1} and for ZnO/Ag-NM the concentrations range between 0.01 to 0.25 mg.ZnO/Ag.L^{-1} (1% Ag).

Combined exposures

To perform the combined exposures the methodology applied in the single exposures was used deferring only in the number of replicates. The number of replicates were decrease to three replicates instead of five for immobilization and decreased to one in the case of the reproduction tests. These reduction allowed an increase of treatments which allows covering a wider range of exposure and it will not prejudice the reliability of the results as the statistics used are based on a regression (for details see below) (Loureiro et al. 2009; Loureiro et al. 2010).

The experimental design for the acute test was based on a full factorial design. For the reproduction test the concentrations were applied in a fixed ray design based on toxic units (TU) (figure 4). One TU was equal to the EC_{50} value of each nanomaterial and the TU never exceeded 2 to avoid mortality. Within each combined experiment an exposure to each chemical alone was performed using the concentrations applied in the single exposures.

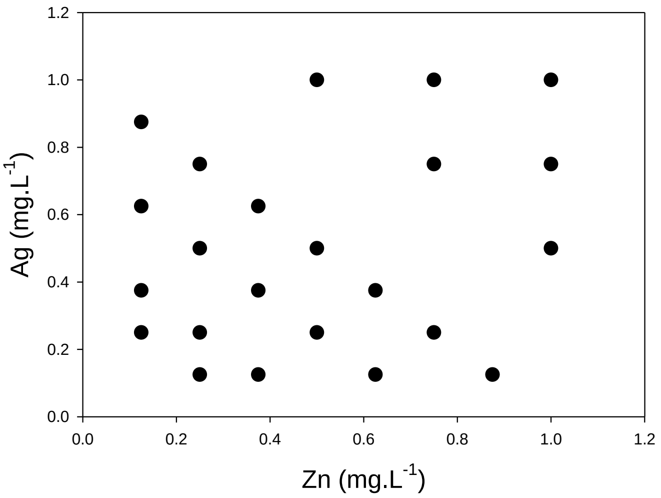


Figure 4. Fixed ray design of the combinations used for the ZnO-NM and Ag-NP mixture chronic toxicity test.

Statistical analysis

The concentration inducing 50% of immobilization (LC_{50}) and 50% of effect (EC_{50}) were calculated using the best fit, by a nonlinear regression using the SigmaPlot for windows version 11 (Systat Software, Inc., 2008).

One-way analysis of variance (ANOVA) was used to identify significant differences between treatments and control ($P < 0.05$) (Systat Software, Inc., 2008). A Dunnett's test was performed to compare each treatment with the control.

The mixture set up was analyzed with the MixTox tool (Jonker et al. 2005). The MixTox tool allows to analyze binary mixtures and uses the conceptual models of Concentration addition (CA) and Independent action (IA) for toxicity prediction. Recently the EFSA advice on the use of the CA model as the most conservative one, therefore it was followed in the present study to predict mixture toxicity. Deviations for synergism (more severe effect) and antagonism (less severe effect) were then evaluated by extending the mathematical equation of CA, by adding parameter *a*. With the addition of parameters *a* and also *b* (another extension to depict changings in patterns) dose-ratio (DR) and dose-level (DL) deviations were assessed. The biological meaning of the positive or negative values of the parameters *a* and *b* can the found in table 1.

To allow further comparison with the mixture of ZnO-NM and Ag-NP, the results from ZnO/Ag-NM were also analysed with the MixTox tool, simulating a mixture toxicity approach.

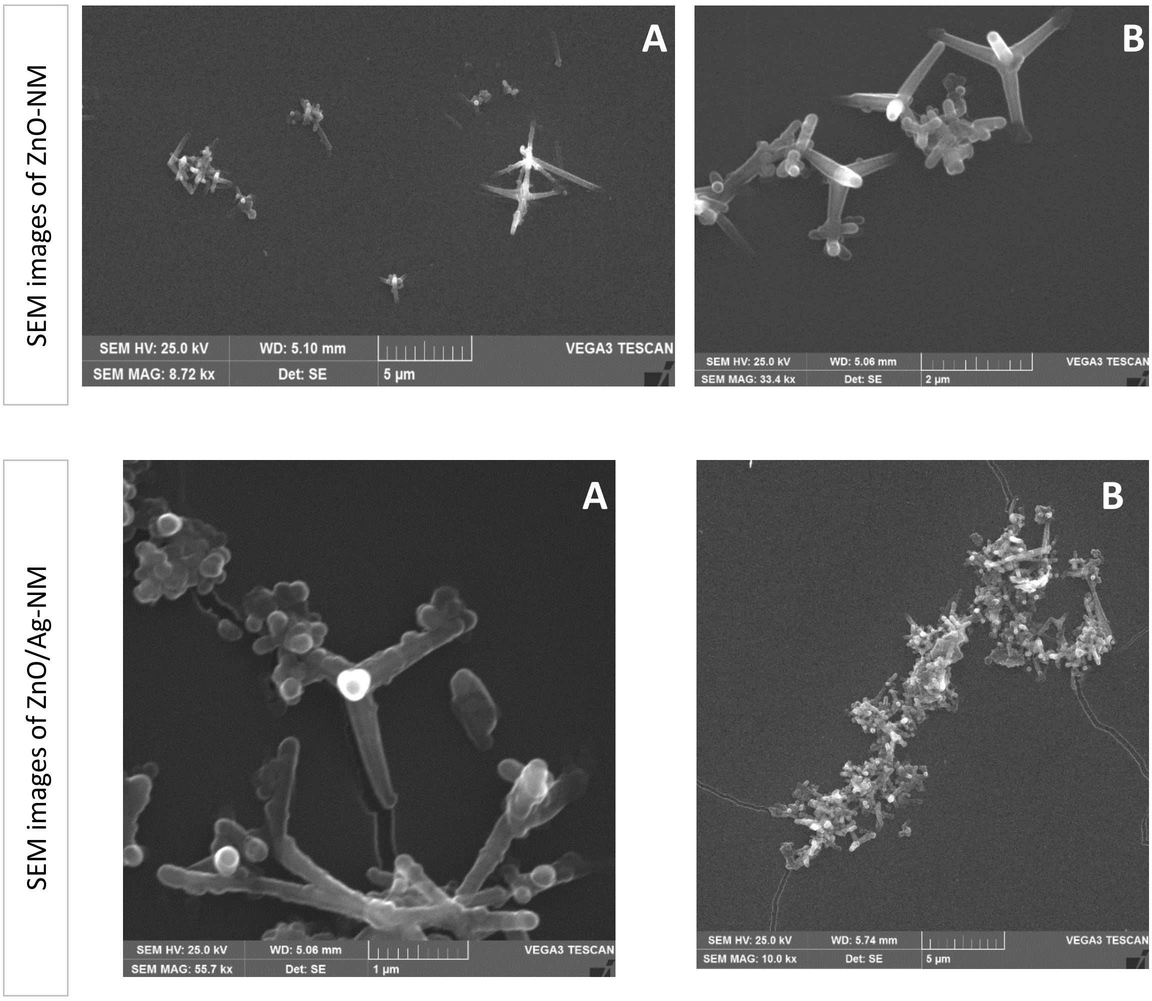
Table 1. Interpretation of additional parameters (*a* and *b*) that define the functional form of deviation patterns from concentration action. Adapted from Jonker et al. (2005)

	Concentration addition	
Deviation patter	**Parameter *a***	**Parameter *b***
Synergism/antagonism	$a > 0$: Antagonism $a < 0$: Synergism	
Dose ratio dependence	$a > 0$: Antagonism, except for those mixture ratios where significant negative *b* indicate synergism $a < 0$: Synergism, except for those mixture ratios where significant positive *b* indicate antagonism	$b_i > 0$ Antagonism where the toxicity of the mixture is caused mainly by toxicant *i* $b_i < 0$ Synergism where the toxicity of the mixture is caused mainly by toxicant *i*
Dose level dependence	$a > 0$: Antagonism low dose level and synergism high dose level $a < 0$: Synergism low dose level and antagonism high dose level	$b_{DL} > 1$: Change at lower EC_{50} level $b_{DL} = 1$: Change at EC_{50} level $0 < b_{DL} < 1$: Change at higher EC_{50} level $b_{DL} < 0$: No change but the magnitude of S/A is DL dependent

2.3. Results

2.3.1. Nanomaterials

Figure 5 presents the SEM and STEM images of the nanomaterials at time zero and after 48h. For both ZnO-NM and ZnO/Ag-NM, it was observed that the nanomaterials had a tetrapod shape and it were present in different sizes. Agglomerates in different sizes were also observed as well as separate particles. Also, for both nanomaterials the appearance did not change after 48h. Ag-NP present disperse particles with a spherical shape and in a size smaller than 10nm, no changes were observed after 48h.



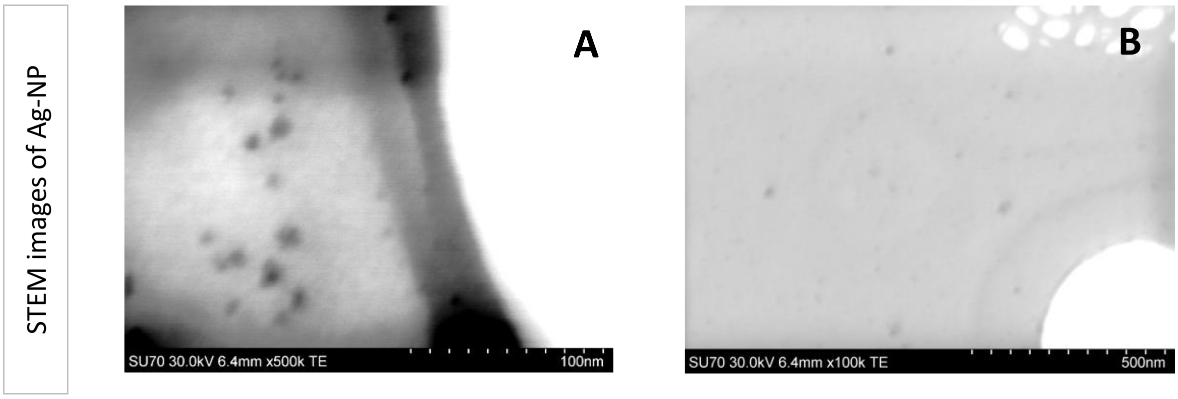


Figure 5. Characterization of nanomaterials using SEM images for ZnO-NM and ZnO/Ag-NM and STEM images for Ag-NP; A – time zero; B – After 48h

2.3.2. Single exposures

Immobilization tests

All the nanomaterials tested showed to increase the mortality of *Daphnia magna* with the increase of concentration applied. For ZnO-NM the 48h-LC_{50} was 1.29 mg.Zn.L^{-1} whereas Ag-NP presented a LC_{50} of 0.09 mg.Ag.L^{-1}. Regarding ZnO/Ag-NM a 48h-LC_{50} of 0.47 mg.ZnO/Ag.L^{-1} was obtained. Values of 48h-LC_{50} in Zn, Ag and ZnO/Ag for each nanomaterial are depicted in table 2.

Table 2. LC_{50} values of the nanomaterials tested presented in mg.Zn.L^{-1}, mg.Ag.L^{-1} and in mg.ZnO/Ag.L^{-1}. Results are expressed as mean ± standard error; R^2 is the coefficient of determination.

	Immobilisation test **48h-LC_{50} (mg.L^{-1})**					
Nanomaterial	**Zn**	**R^2**	**Ag**	**R^2**	**ZnO/Ag**	**R^2**
ZnO-NM	1.29 ± 2.15	0.90	-		-	
Ag-NP	-		0.09 ± 5.83	0.98	-	
ZnO/Ag-NM	0.47[a]		0.006[b]		0.59 ± 0.02	0.84

[a] equivalent values of Zn for ZnO/Ag-NM; [b] equivalent values of Ag for ZnO/Ag-NM

Reproduction tests

The EC_{50} values regarding the reproduction tests for ZnO-NM, Ag-NP and ZnO/Ag-NM can be found in table 3. At the end of all the reproduction tests, mortality of parental control animals was always less than 20% and the mean number of live offspring was always higher than 60 neonates per parental organisms, therefore validating all the tests (OECD 1998). Also the measured parameters validated our results with dissolved oxygen ranging between 5.13 and 9.07 mg.L^{-1}, conductivity between 500 and 630 µS/cm and pH between 7.66 and 8.36.

Table 3. EC_{50} values of the nanomaterials tested presented in mg.Zn.L-1, mg.Ag.L-1 and in mg.ZnO/Ag.L-1. Results are expressed as mean ± standard error; R^2 is the coefficient of determination.

	Reproduction test **21d-EC_{50} (mg.L^{-1})**					
Nanomaterial	**Zn**	**R^2**	**Ag**	**R^2**	**ZnO/Ag**	**R^2**
ZnO-NM	0.25 ± 0.01	0.89	-		-	
Ag-NP	-		0.54 ± 0.04*	0.68	-	
ZnO/Ag-NM	0.16 [a]		0.002 [b]		0.20 ± 0.01	0.72

[a] equivalent values of Zn for ZnO/Ag-NM; [b] equivalent values of Ag for ZnO/Ag-NM, *value extrapolated as it was higher than the highest concentration used.

For ZnO-NM significant differences in the number of neonates produced during the 21 days were observed at concentrations of 0.2, 0.3 and 0.4 mg.Zn.L^{-1} (figure 6-A) (one way ANOVA, $F_{4,41}$ = 83.86, p≤0.001, Dunnett's method, p<0.05). Significant differences on the daphnids' length was observed at all concentrations used in the test (figure 6-B) (one way ANOVA, $F_{4,41}$ =38.37, p≤0.001, Dunnett's method, p<0.05).

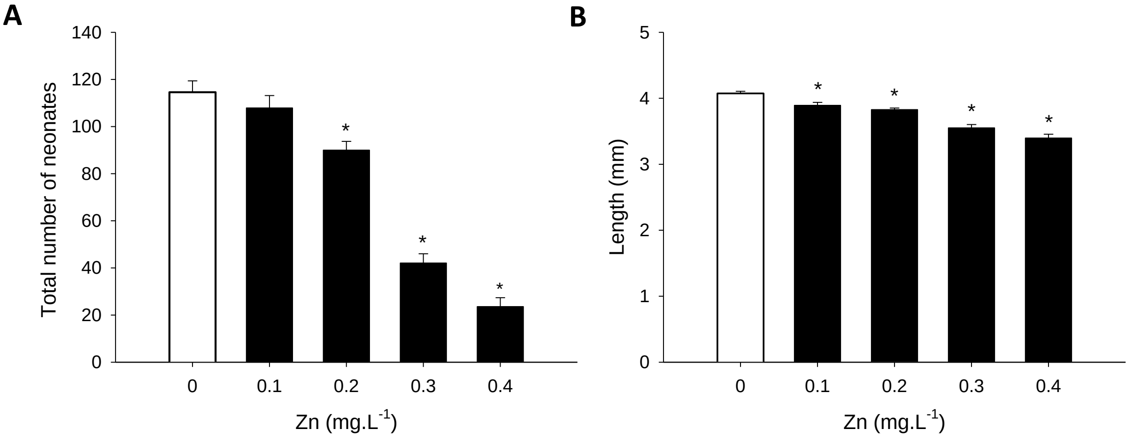


Figure 6. Total number of neonates per daphnia (A) and daphnids' length (mm) (B) after a 21 day exposure to ZnO-NM. Data is expressed as $mg.Zn.L^{-1}$ mean values ± st. error. * p<0.05, Dunnett test.

In the reproduction test with Ag-NP a significantly decrease in the mean number of neonates produced per daphnia during the 21 days (figure 7-A) was observed at the three highest concentrations (0.3, 0.4, 0.5 $mg.Ag.L^{-1}$) (one way ANOVA, $F_{5,48} = 21.04$, $p \leq 0.001$, Dunnett's method, $p<0.05$). No significant differences were observed for the length of the daphnids (figure 7-B), showing that Ag-NP do not affect the growth of the daphnids when compare with the control at the Ag levels used.

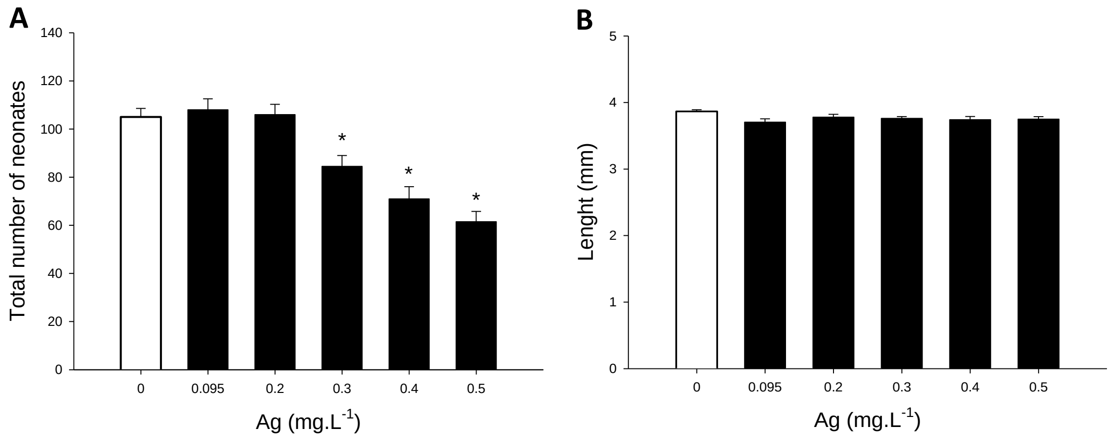


Figure 7. Total number of neonates per daphnia (A) and daphnids' length (mm) (B) after a 21 day exposure to Ag-NP. Data is expressed as $mg.Ag.L^{-1}$ mean values ± st. error. * p<0.05, Dunnett test.

Alterations in the number of offspring and in the length of daphnids were observed in exposures to ZnO/Ag-NM. The cumulative number of neonates produced per daphnia (figure 8-A) significantly decreased in the two highest concentrations (0.1 and 0.2 $mg.Zn.L^{-1}$) (one way ANOVA,

$F_{5,50}$ = 30.59, p≤0.001, Dunnett's method, p<0.05). The length of the adult daphnids was significantly affected at concentrations of 0.05, 0.1 and 0.2 mg.Zn.L^{-1} (figure 8-B) (one way ANOVA, $F_{5,50}$ = 10.95, p≤0.001, Dunnett's method, p<0.05).

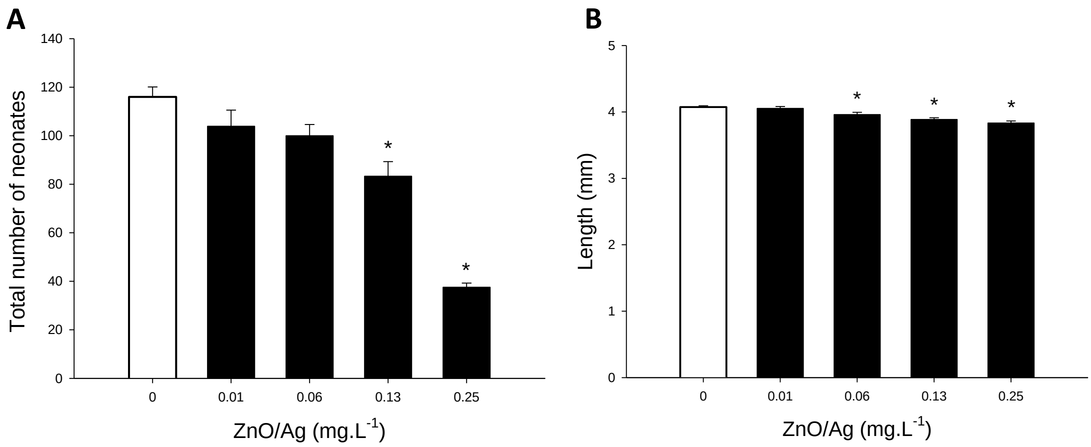


Figure 8. Total number of neonates per daphnia (A) and daphnids' length (mm) (B) after a 21 day exposure to ZnO/Ag-NM. Data is expressed as mg.ZnO/Ag.L^{-1} mean values ± st. error. * p<0.05, Dunnett test.

2.3.3. Combined exposures approach

The LC_{50} values for the single exposures to ZnO-NM and Ag-NP performed during the combine exposures were 0.66 mg.Zn.L^{-1} (st.error=0.06; r^2=0.64) and 0.09 mg.Ag.L^{-1} (st.error=0.06; r^2=0.64), respectively.

For the mixture of ZnO-NM and Ag-NP the results fitted the CA model (SS=84.82; r^2=0.78; p=2.11x10^{-63}) and showed a deviation pattern for dose-level (SS=70.61; r^2=0.82; p=8x10^{-4}; a=-2.63; b=0.70), as showed in figure 9. The negative *a* and the positive *b* indicate synergism at low dose levels that change to antagonism when the effects are higher than 50%.

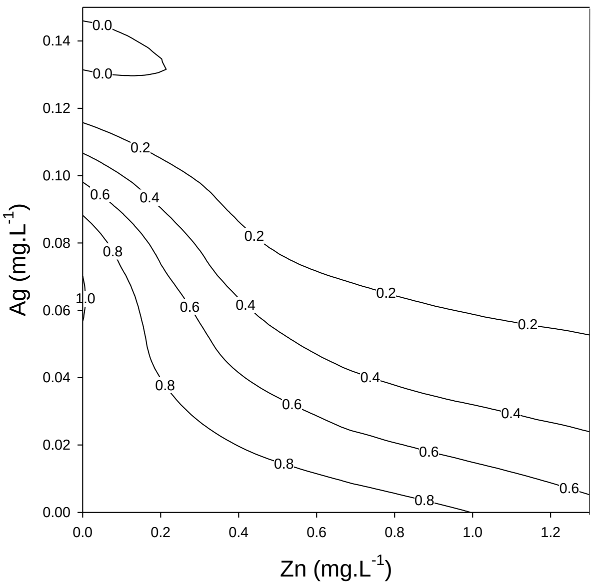


Figure 9. Dose-response pattern for the 48h combined exposure of *Daphnia magna* to ZnO-NM and Ag-NP for the immobilization, showing dose-levels deviations from the CA model: synergism occur at low concentrations changing to antagonism at dose levels higher than the EC_{50}.

The mixture toxicity concentrations for the reproduction test were design in a fixed ray based in toxic units. Although the toxic units never exceed 2 to avoid mortality, mortality was observed in thirteen combinations as shown in figure 10 by the white circles.

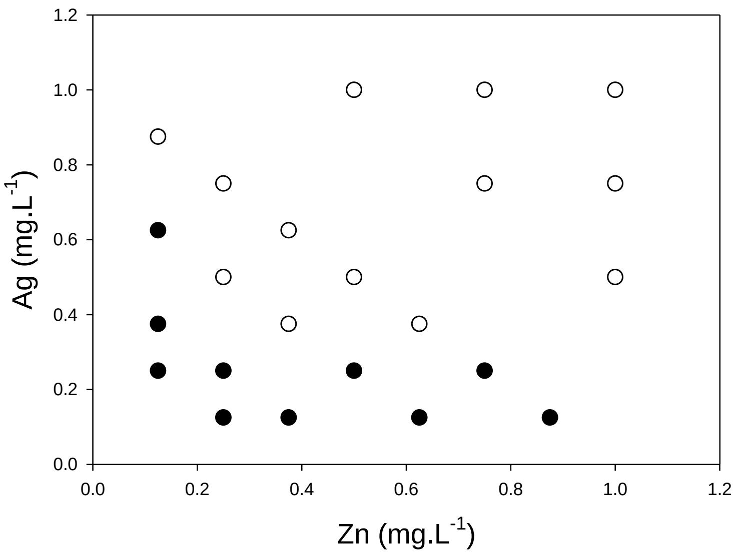


Figure 10. Fixed ray design used in the reproduction test showing the combinations that lead to mortality (white circles) and those that reach the end of the test, where data (number of neonates) was recorded.

The single exposures performed during the combined exposures derived EC_{50} values of 0.24 mg.Zn.L^{-1} (st.error=0.02; r^2=0.94) for ZnO-NM and 0.52 mg.Ag.L^{-1} (st.error=0.04; r^2=0.80) for Ag-NP. The combined exposures fitted the CA model (SS=5674.34; r^2=0.65; p=0.005), which was afterwards improved by the equation extension to synergism (SS=2101.70; r^2=0.87; p=2.4x10^{-5}; a=-2.43) (figure 11).

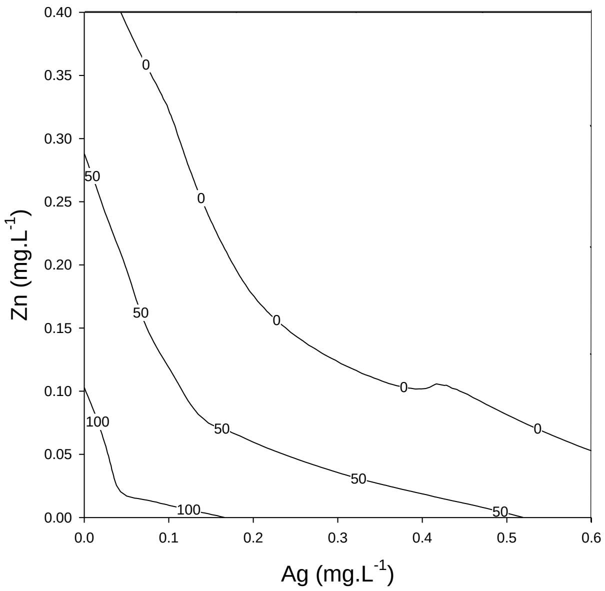


Figure 11. Dose-response pattern for the 21 days combined exposure of *Daphnia magna* to ZnO-NM and Ag-NP for the reproduction, showing synergism from de CA model.

The results of ZnO/Ag-NM were also modeled with concentration addition (CA) reference model to predict patterns for combined exposures, based on the extrapolated concentrations for both ZnO and Ag. Although the results fitted the CA model only 36% of the data was well fitted (SS=215.80; r^2=0.36; p=2.11x10^{-25}). After the addition of parameter a and b to the CA equation the data fit was improved and a DR deviation obtained (SS=70.61; r_2=0.98; p=1.04x10^{-44}; a=-125.06, b=135.44). The interpretation of the parameters showed that synergism occurs when the mixture is compose by a high concentration of Ag-NP and low concentrations of ZnO-NM. The pattern changes to antagonism when the inverse is observed, i.e. high concentrations of ZnO-NM and low concentrations of Ag-NP.

The results from the reproduction of ZnO/Ag-NM exposures fitted the CA model (SS=1829.07; r^2=0.84; p=1.2x10-3) and after the addition of parameters a and b the dose-level deviation was the one with the best fit (SS=203.35; r^2=0.98; p=0.03; a=-157.5, b=0.73). The interpretation of the parameters showed that synergism occur at low concentrations and that it changes to antagonism at dose levels higher than the EC_{50}.

2.4. Discussion

The aim of this work was to understand if it is possible to predict the toxicity of ZnO/Ag-NM based on the toxicity of its components, ZnO-NM and Ag-NP. In addition, another aim was to infer if the toxicity of this nanomaterial will be similar to the toxicity of an "artificial" mixture of its components. For that single exposures as well as mixture exposures were performed with ZnO-NM and Ag-NM and the toxicity patterns compared to the one for ZnO/Ag-NM.

For ZnO-NM, negative effects were observed in the survival, reproduction and growth of *Daphnia magna* with the increasing concentration of ZnO-NM. Despite the tetrapod shape of the nanomaterials, the LC_{50} (1.29 mg.Zn.L^{-1}) and EC_{50} (0.25 mg.Zn.L^{-1}) values were in the range of the ones found in the literature for spherical nanomaterials. The values found for *Daphnia magna* ranged from 0.89 to 3.2 mg.ZnO.L^{-1} for immobilization (Ma et al. 2013; Lopes et al. 2014) and from 0.26 to 0.36 mg.L^{-1} for reproduction (Lopes et al. 2014). Since ZnO-NM toxicity can be related to ion dissolution the similarity of results can be due to the toxicity of Zn ions. According to the SEM images, the ZnO tetrapods used in our study presented different sizes. Some studies regarding the toxicity of ZnO-NM state that the size play an important role in the toxicity, but this is not consensual. Lopes et al. (2014) observed that no significant differences were obtained when comparing the toxicity two ZnO-NP and a microsized form of ZnO (30nm, 50-70nm and >200nm) in *Daphnia magna*. A different results was obtained by Heinlaan et al. (2008) where the toxicity of the ZnO bulk material was 3-fold higher than for the ZnO-NP. Differences in the results can be due to aggregation of the NMs which can alter their dissolution rate and therefore their toxicity.

For the immobilization, Ag-NPs showed higher toxicity when compared to ZnO-NM, presenting a LC_{50} (0.09 mg.Ag.L^{-1}) more than ten times lower than the one for ZnO-NM (1.29 mg.Zn.L^{-1}). Studies found in the literature regarding the Ag-NPs toxicity show a large variety of results, due to high variability in the characteristics of the particles (e.g. coated or uncoated) and in the dispersion of the nanomaterials (e.g. colloids or suspensions). Colloids are usually prepared with coated nanomaterials and they are normally more stable than the suspensions. Ag-NPs suspensions, as the one used in this study, tend to have a high tendency to aggregate which alter their surface area to volume ratio and they also exhibit high sedimentation ratios (Asghari et al. 2012). These characteristics alter their toxicity and much higher LC_{50} values for the suspensions can be found in the literature when compared to the colloids. Asgahri et al. (2012) compared the toxicity of two Ag-NPs colloids and a Ag-NP suspension and found that Ag-NP suspensions had a LC_{50} of 0.187mg.L^{-1} which was much higher than the values for the colloids (0.002 and 0.004 mg.L^{-1}). The presence of food can also alter the toxicity of Ag-NPs. Gaiser et al. (2011) tested the toxicity of Ag-NP suspension to *Daphnia magna* during 96h and obtained a LC_{50} of 0.1mg.L^{-1}. This value is similar to ours despite the differences in the duration of the test. This can be explained by the feeding of the organisms during the entire experiment, which can lead to an increase on their fitness and to a decrease in the toxicity. The presence of food is known to decrease the toxicity of Ag-NPs to *Daphnia magna* (Ribeiro et al. 2014; Mackevica et al. 2015). Ribeiro et al. (2014) showed that Ag-NPs do not enter the algae cells but rather they attach to the surface of the algae. This may lead to an increase in the sedimentation and a decrease of Ag-NPs available for the organisms (Mackevica et al. 2015). The presence of food also seems to play an important role in the toxicity of Ag-NPs used in our tests since we obtained an EC_{50} (0.54 mg.Ag.L^{-1}) higher than the LC_{50} (0.09 mg.Ag.L^{-1}) without food, which is normally not obtained. No significant decrease in the size of the organisms was observed at the end of the test.

The LC_{50} and EC_{50} values for the single exposures carried out simultaneously with the mixture trials, for both ZnO-NM and Ag-NP, were similar to the values obtained during the first tests (used to design the experimental set up of the mixture trials). These results validate our tests and organisms sensitivity and shows that the reduction of replicates did not affect the results. To predict the mixture toxicity of ZnO-NM and Ag-NP, the CA model was used as it has been advised by the EFSA report (European Food Safety Authority 2015). Both, immobilization and reproduction, presented deviations from the CA model. To the immobilization, a synergistic pattern can be observed for low concentrations that changes to antagonism at concentrations higher than the EC_{50}. The antagonistic response may be a result of aggregation of the nanomaterials due to high number of particles in the mixture, leading possibly to sedimentation and therefore decreasing toxicity. Since the predicted environmental concentrations in surface waters for ZnO and Ag nanomaterials are in the range of the ng.L^{-1} (Gottschalk et al. 2009), the most probable scenario to occur in the environment is the synergistic response. The synergistic pattern for immobilization at low concentrations can also be observed in the reproduction test. Although the toxic units never exceed 2 with the purpose of avoiding mortality, mortality occurred in thirteen concentrations of twenty three. For the surviving organisms, a synergistic response was observed for the number of neonates produced. Despite this synergistic pattern in reproduction, the observed mortality indicates also an increase in toxicity more than expected from the single exposure trials. Some studies related the toxicity of ZnO-NM and Ag-NM to the release of Zn and Ag ions (Adam et al. 2014; Jo et al. 2012). The presence of Zn^{+} and Ag^{+} may have negative impacts in the uptake of ions that are essential to the good health of the organisms. Zn is an essential metal but it can have negative effects if present at high concentrations. Zn ions can compete with Ca^{2+} which can lead to a disturbance in the Ca content in the body (Muyssen et al. 2006). A reduction of Ca content in *Daphnia magna* can negatively affect the movement and filtration rate which will eventually lead to a reduction of growth and reproduction due to feeding impairment (Muyssen et al. 2006). Ag^{+} present in the media can lead to ion deregulation due to the competition with Na^{+} uptake (Bianchini & Wood 2002). Ion deregulation will eventually lead to the dead of the organisms.

Very few studies determine the toxic effects of exposures with more than one nanomaterial. Zhao et al. (2012) observed a synergistic effect to the survival and reproduction of *Daphnia magna* when combining CuO-NM and ZnO-NM. A different response is observed when the species *Phaseolus vulgaris* (common bean) is exposed to a mixture of CuO-NM and ZnO-NM, where higher toxicity was observed to exposures only to CuO-NM (Dimkpa et al. 2015).

The nanomaterials composed of ZnO nanomaterials with Ag-NPs, in a percentage of 1%, on its surface, presented lower LC_{50} and EC_{50} values than the ones of the ZnO-NM. When looking at the results in Zn concentration the values for the LC_{50} and EC_{50} decreased in more than 2-fold for the ZnO/Ag-NM when compared to the ZnO-NM alone. In addition, and based on the principal of the CA model and looking at Table 3, to have a 50% of effect on the reproductive output (eq. to 1 TU), one would need a mixture with a concentration of 0.15 mg $Zn.L^{-1}$ (eq. to the EC_{25} or ½ TU) jointly with a concentration of 0.27 mg Ag $.L^{-1}$ (eq. to the EC_{25} or ½ TU). From Table 3, while relating the effective concentration of ZnO/Ag to their components, the concentration of ZnO/Ag inducing 50% of effects was composed by 0.16 mg $Zn.L^{-1}$ but with a much lower concentration of Ag, which represented 1% of the mass (0.002 mg Ag $.L^{-1}$). This clearly indicates that less Ag was needed in the decorated ZnO to induce the effects predicted.

Metal nanomaterials toxicity is usually linked to oxidative stress (Chang et al. 2012). NMs can cause oxidative stress by eliminating antioxidants or by direct production of ROS (Sánchez et al. 2011). Since the addition of Ag-NPs to the surface of ZnO-NM will increase the photocatalytic activity, this may lead to an increase in the ROS production when compared to the production by the nanomaterials alone. Oxidative stress can lead to membrane damage, lipid peroxidation or protein denaturation (Manke et al. 2013). When analyzing the results with the MixTox tool a dose-ratio deviation can be observed for immobilization and a dose-level deviation for the reproduction. For the immobilization a synergistic response can occur at high concentrations of Ag-NP and low concentrations of ZnO-NM but it changes to antagonism if the opposite is observed. For reproduction it was observed a synergistic pattern at low concentrations and an antagonistic pattern at concentrations higher than the EC_{50}. The increase concentration of Ag-NPs in the surface of the ZnO-NM will increase the photocatalytic activity as demonstrated by Ren et al. (2010). These may lead to an increase of ROS production and therefore an increase in the toxicity. Li et al. (2010) observed that the nanomaterials composed by Ag and gold (Au) in different percentages had different toxicity to *Daphnia magna*. The nanomaterials containing Ag in a percentage of 80% and 20% of Au were less toxic than expected, demonstrating that Au can reduce the toxicity of Ag-NM. When the ratio of Ag and Au is inverse (20% of Ag and 80% of Au) the nanomaterials were more toxic than expected (Li et al. 2010).

2.5. Conclusion

Our results showed that the toxicity mixture approach did not accurately predict the toxicity of the heteronanostructure used in the present study. In addition, the CA conceptual model was not the best model to predict ZnO and Ag joint toxicity. The mixture of ZnO-NM and Ag-NP did not show an additive pattern but rather deviations such as dose-level and synergism. Since nanomaterials in the environment can be in a mixture with other nanomaterials it is important to understand how they will behave and more studies regarding the mixture toxicity of NMs need to be addressed.

Also, ZnO-NM decorated with Ag-NP on its surface showed higher toxicity when compare with the predicted toxicity based on the results from the individual components. The toxicity of these new nanomaterials needs to be address as a single material and not based on the toxicity of the single components.

In conclusion, more studies regarding mixture toxicity of NMs need to be addressed, and the hazard of new nanomaterials evaluated before they are introduced in the market since their toxicity cannot be predicted by the single components' toxicity.

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Chapter III

General discussion and conclusions

3. General discussion and conclusions

3.1. General discussion and conclusions

With the increasing production and application of NMs in several products, it is likely they will end up in the environment where they may cause harmful effects to the organisms (Mitrano et al. 2015; Bhatt & Tripathi 2010). Due to the different characteristics of the NMs (e.g. chemical composition, surface charge and dissolution rate) as well as different characteristics of the environment (e.g. pH, OM, IS) predicting their behavior in the environment is very difficult (Handy et al. 2008; Markus et al. 2015; Batley et al. 2013). Since the aquatic environment can be the final destination of the NMs many studies have focus on evaluating their toxicity to aquatic organisms (Choi et al. 2014; Lapresta-Fernández et al. 2012; Skjolding et al. 2014).

When in the environment NMs will probably be in a mixture with other chemicals which may change the overall toxicity. Despite this fact, only few studies have evaluated their interaction (Dimkpa et al. 2015; Zhao et al. 2012). Moreover, some NMs are being combined with other NMs (Mageshwari et al. 2015; Wang et al. 2012; Geetha et al. 2015), to improve their functions, which can also end up in the environment. It is crucial to understand NM-NM interaction and how they will behave and their inherent hazard.

This study aimed at evaluating if the toxicity of a new NM formed by ZnO-NM with 1% of Ag-NP on its surface can be predicted by the toxicity of the single components or/and by mixture toxicity concepts. The effects of survival and reproduction to *Daphnia magna* were assessed for ZnO-NM, Ag-NP, ZnO/Ag-NM and for a mixture of ZnO-NM and Ag-NPs.

Both, ZnO-NM and Ag-NP, showed increasing mortality with the increase of concentrations and also negative effects to the reproduction with a decreasing number of neonates with increasing concentrations. Only ZnO-NM showed to affect daphnids growth after the 21 day exposure period. ZnO-NM and Ag-NP toxicity is mainly attributed to the dissolution of ions which may lead to ion deregulation and also to oxidative stress due to ROS production (Sánchez et al. 2011; Miao et al. 2010).

The results from the mixture and from the ZnO/Ag-NM toxicities were analyzed using the CA model. Both showed deviations from the model demonstrating that there is an interaction between the two components. Regarding the mixture, a dose-level deviation from the conceptual model was observed for immobilization and synergism for the reproduction. Whereas for ZnO/Ag-

NM the best fit for the survival was the dose-ratio deviation and for the reproduction a dose-level deviation was observed.

Based on the results it can be conclude that both, the mixture and the ZnO/Ag-NM, will not behave based on their components toxicity and most likely a synergistic pattern will be observed when in the aquatic environment.

These results highlight the importance of taking into account the present of other NMs when evaluating NMs toxicity in risk assessment studies. Moreover, the toxicity of new nanomaterials should be addressed also as a single nanomaterial and not based on the toxicity of its components.

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