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Chronic effects of silica nanoparticles in *Vibrio fischeri, Raphidocelis subcaptata, Danio rerio* and *Allium cepa*

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To my wonderful family and

to my amazing friends

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“We cannot solve our problems with the same thinking we used when we created them”

(Albert Einstein | 1879 – 1955)

ABSTRACT

SILVA, G. H. **Chronic effects of silica nanoparticles in *Vibrio fischeri, Raphidocelis subcaptata, Danio rerio* and *Allium cepa.*** 2014. 56 f. Dissertação (Mestrado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2014.

Scientific research using nanotechnology is a relatively recent development with a variety of potential applications in many fields of science. Within this field of research, many new products, with improved performances, have been developed. Despite increased research on its toxicity to ecosystem, the knowledge about this area is still limited. To evaluate the toxicity and genotoxicity of different sizes silica nanoparticles (SiNP) to the environment, different species, on different trophic levels (*Vibrio fisheri, Raphidocelis subcapitata, Danio rerio* and *Allium cepa*) were exposed to TM40 (22 nm), HS30 (12 nm), SM30 (7 nm) with concentrations ranging from 0.19 to 163.8 g/L (TM40) and 0.29 to 122.85 g/L (HS30 and SM30), and the following parameters were monitored during exposure: production of bioluminescence (*V. fischeri*), growth rate (*R. subcapitata*), embryonic development and DNA damage (*D. rerio*) and germination rate, growth and DNA damage (*A. cepa*). Within each test SiNP present a size dependent chronic toxicity. The bioluminescence test present a EC50 of 29.11, 32.34 and 4.58 g/L for TM40, HS30 and SM30, respectively. For the growth rate assay the EC50 was 9.32, 9.07 and 7.93 g/L for TM40, HS30 and SM30, respectively. And for the zebra fish embryonic development test for TM40, HS30 and SM30, the EC50 was 5.85, 1.13 and 2.68 g/L respectively. All particles also induce phytotoxicity in *A. cepa*, growth and germination reduce significatively when expose to SiNP. Futhermore genotoxic effects were also induced by the particles for both *A. cepa* and *D. rerio*. Therefore, SiNP can cause toxicity to the environment and size can strongly influence this toxicity.

Keywords: Nanotoxicology. Nanoparticles. Bioluminescence. Growth rate. Embryonic development DNA damage.

RESUMO

SILVA, G. H. **Efeitos crônicos das nanopartículas de sílica em *Vibrio fischeri, Raphidocelis subcaptata, Danio rerio* e *Allium cepa.*** 2014. 56 f. Dissertação (Mestrado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2014.

Com uma variedade de aplicações potenciais, em diversos campos da ciência, as pesquisas científicas utilizando nanotecnologia são de desenvolvimento relativamente recente. Dentro deste campo de pesquisa, vários novos produtos, com desempenhos melhorados têm sido desenvolvidos. Apesar do aumento de pesquisas sobre a toxicidade dessas tecnologias à biota, o conhecimento sobre esta área ainda é limitado. Visando avaliar a toxicidade e genotoxicidade de nanopartículas de sílica (SiNP) no meio ambiente diferentes espécies pertencentes a diversos níveis tróficos (*Vibrio fisheri*, *Raphidocelis subcapitata*, *Danio rerio* and *Allium cepa*) foram expostos a Ludox TM40 (22 nm), Ludox HS30 (12 nm) e Ludox SM30 (7 nm). As espécies de teste foram expostas a concentrações de nanopartículas (NP) variando de 0.29 a 163.8 g/L (TM40) e 0.19 a 122.85 g/L (HS30 e SM30) e os seguintes parâmetros monitorizados durante a exposição: a produção de bioluminescência (*V. fischeri*), o crescimento taxa (*R. subcapitata*), inibição de alimentação (*D. magna*), desenvolvimento embrionário e dano ao DNA (*D. rerio*) e taxa de germinação, crescimento e danos ao DNA (*A. cepa*). Nos testes feitos com as SiNP foi observado que a toxicidade é dependente do tamanho da partícula. O ensaio de bioluminescência apresentou um EC50 de 29.11, 32.34 e 4.58 g/L para TM40, HS30 e SM30, respectivamente. Para o ensaio de taxa de crescimento o EC50 foi 9.32, 9.07 e 7.93 g/L para TM40, HS30 e SM30, respectivamente. E para o teste de desenvolvimento embrionário com peixe zebra, para o TM40, HS30 e SM30 o EC50 foi de 5.85, 1.13 e 2.68 g/L, respectivamente. Todas as partículas também induziram fitotoxicidade em *A. cepa*, crescimento e germinação reduziram significativamente quando o organismo foi exposto a SiNP. Efeitos genotóxicos também foram induzir pelas partículas, tanto para *A. cepa* quanto para *D. rerio*. Portanto, as SiNP podem causar toxicidade ao ambiente e o tamanho pode influenciar fortemente a essa toxicidade.

Palavras-chave: Nanotoxicologia. Nanopartículas. Bioluminescência. Taxa de crescimento. Desenvolvimento embrionário. Dano ao DNA.

SUMMARY

1. INTRODUCTION

In the late twentieth century nanotechnology, the production of particulate materials with at least one dimension smaller than 100 nm, emerged (CHRISTIAN et al., 2008). This technology is growing rapidly, producing new products and receiving increasing investments (CUNNINGHAM; PORTER, 2011), including in Brazil (KAY; SHAPIRA, 2011) and with it a multitude of applications and products being produced, with medicinal to environmental applications (ROCO, 2011).

More than 900 products based on nanomaterials are already on the market (CHATTERJEE, 2008), many are already available even for everyday use. In most sunscreens are nanoparticles (NP) of approximately 10 nm that are used to absorb ultraviolet (UV) radiation. It is estimated that approximately 1000 tons of TiO2 NP (14 nm) and ZnO (30-200 nm) are produced for this purpose (NOHYNEK et al., 2007). One of the most widely used nanomaterials today is nanosilver (nano-Ag), it has a very high bactericidal activity and is therefore incorporated in different materials, especially in the medical field, being used in tissue, implants, toothpaste, shoes, containers for storing food, and many others (PASCHOALINO, 2010; CHEN; SCHLUESENER, 2008). Some NP has redox and/or semiconductor properties that can be used in the remediation of polluted environments (QUINA, 2004).

This technology brings major advances and promises to improve the quality of life and of the environment, however, due to the large number of new products using this technology, its use has been widely debated as to potential risks such as environmental contamination that may present unknown or high toxicity. According to Klaine et al. (2008) the potential release into the environment and the subsequent ecosystem pollution is becoming a growing concern. It is known, for example, that nanoAg can be inhaled and accumulate into the brain, and if absorbed by the skin can cause damage to critical structures such as mitochondria (CHEN; SCHLUESENER, 2008).

The use of this technology can pose significant risks because of the difficulty to assess their possible toxicological effects. According to Dhawan and Parmar (2009), the mechanism of interaction between nanomaterials (NM) and biological systems depend on the size, shape, composition and surface properties of the NM in question.

A new area of toxicology is emerging to monitor the development of this technology, the nanotoxicology, which evaluated the deleterious effects that NM may have on humans and on the environment. According to Stone et al. (2010), the nanotoxicology refers to the study of the potential harmful effects of NM and NP. The risks that the NM and NP can lead to aquatic and/or terrestrial environments are not know yet, so the concern about nanotoxicity is rising as new products are being synthesized, handling and disposal, which can be launched on natural environments without control or regulation (ROSSETTO, 2012).

There are numerous applications for silica nanoparticles (SiNP) and it can be used in many fields, such as chemical industries, biomarkers in medicine, cosmetics, paints, food and medicine (WEI et al., 2010; FEDE et al., 2012). Several studies have shown that these particles can cause damage to human cells (LU et al., 2011; LIN et al., 2006; EOM; CHOI, 2009; SUN et al., 2011; PASSAGNE et al., 2012), complications on pregnancy (YAMASHITA et al., 2011), inhibition of DNA replication and transcription (CHEN; VON MIKECZ, 2005), and heart and lung injury in rats (CHEN et al., 2008). The effect of these particles on human health is well studied, however there are little information about their effect on the environment. With the increasing application of these NP in several areas the chance of these come into direct contact with the environment (mainly water) is rising, so the toxicity of new NM and NP in the environment must be identified for safer use.

1.1. Hypothesis

This Study present sought to test hypothesis that when transform into nanoparticles silica change its properties and cause toxicity to the organisms, this duo the facility that small particles has to entry the organism biological systems.

1.2. Objectives

The general objective of this thesis was to assess the effect of different sizes SiNP in the ecosystem using different organisms for the evaluation and compared the results to evaluate if different sizes equal different toxicity levels.

To achieve the objectives were used toxicity tests measuring bioluminescence, reproduction, growth, germination and death with *Vibrio fischeri, R. subcapitata, Danio rerio* and *Allium cepa*, and genotoxicity tests with *Danio rerio* and *Allium cepa*.

1.3. Structure of the thesis

This thesis comprises an introductory initial text followed by three studies presented in scientific manuscript format written in English language.

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2. CITOTOXICITY AND PHYTOTOXICITY OF SILICA NANOPARTICLES WITH DIFFERENT SIZES TO ROOT TIP CELLS OF *ALLIUM CEPA*

Abstract

Nanotechnology is rapidly expanding, and several new products (nanoparticles-NP) with improve performances have been developed. The Nanoparticles (NP) environmental fate and toxicity is poorly known and needs to be studied for a safe use of this technology. This study aims to investigate genotoxic and cytotoxic impacts of silica nanoparticles (SiNP), with three different sizes, using root tip cells of *Allium cepa* as an indicator organism. *A. cepa* root tip cells were treated with six different concentrations (TM40: 24.43 to 3.2 gL^{-1}; HS30 and SM30: 18.22 to 2.4 gL^{-1}) of three engineered SiNP (TM40 – 22nm; HS30 – 12nm; SM30 – 7nm) dispersion. Measured endpoints were as the following: mitotic index, different types of chromosomal aberrations, disturbed metaphase, sticky chromosome, breaks and micronucleus. No chromosomal aberration was observed in the negative control and the mitotic index (MI) value was 16.6%. With increasing concentration of NP a decrease in the mitotic index were observed, there was significant decrease of the MI for all the tested NP and for all concentrations. For all NP and all concentrations (except for TM40 and 5.4 g/L for HS30) cytological effects including the chromosomal aberration were observed in treated cells. Phytotoxic effects were also observed, germination rate and root growth presented significant values ($p<0,05$, Kruskal wallis) for all SiNP. Plants are an essential component of the ecosystems so those findings suggest that they need to be included when evaluating the toxicological impact of the NP on the environment.

Keywords: Nanotoxicity. DNA damage. Size-influence.

2.1. Introduction

Nanotechnology has been growing exponentially nowadays and there have been studies in all areas, physical, chemicals, environmental and medicinal. It is expected that on the next decade over 100.000 tons of nanotechnology materials will be produced (ROCO, 1999). Despite many researches about nanoparticles (NP) and nanotechnology there are,'t much about the effects of these nanomaterials on the health and especially on environmental impacts. Nanoparticles, material that has between 1 to 100 nanometer, have shown special toxicity and are usually more toxic than the bulk material of larger size (DONALDSON et al., 2001). SiNP have been studied recently and extensive applications have been found for these particles, it can be used as additives to drugs, chemical industry, printer toners, cosmetics and food (FEDE et al., 2012). Although many studies have shown the cytotoxicity of silica nanoparticles (SiNP) in vitro and in vivo (NAPIERSKA et al., 2009; YE et al., 2010; LU et al., 2011; PASSAGNE et al., 2012; AHMAD et al., 2012) none has used plants as indicator organism.

Plants have been used as indicator organisms for many years, in studies about mutagenesis in higher eukaryotes. The international Programme on Chemical Safety (IPCS, WHO) and the United Nations Environmental Programme (UNEP) validated the *Allium cepa* root chromosomal aberration assay as an efficient and standard test for the chemical screening and in situ monitoring for genotoxicity of environmental substances (CABRERA; RODRIGUEZ, 1999). Rank and Nielsen (1994) observed correlation of 82% between *Allium cepa* tests and carcinogenicity tests on rats. Other studies also showed important correlation between tests with plants and mammals (VINCENTINI et al., 2001; TEIXEIRA et al., 2003; FACHINETTO et al., 2007; RAY et al., 2013), those papers shown that tests with plants can be robust and exposed the dangerous effects that chemicals, such as NP, can have not just on the environment, but also on human health.

The effects of SiNP has been studied on human cells, algae, and fishes (FEDE et al., 2012; VAN HOECKE et al., 2008; FENT et al., 2010) but data on potential toxicity of NP on ecological terrestrial test species is still limited (USEPA, 2007).

Biological effects of SiNP have not been well studied, and there are still many unresolved issues and challenges to be evaluated. Therefore, the present study is designed to investigate cytotoxic and genotoxic impacts of SiNP on *A. cepa*.

2.2. Material and Methods

2.2.1. Studied NP

SiNP, electrostatically stabilized with negative charges and with sodium as counterion and with different sizes were studied in the present work: Ludox TM40 (size: 22 nm), Ludox HS30 (size: 12 nm) and Ludox SM30 (size: 7 nm) (supplied by the chemical department of the coimbra university). Stock suspensions of 400 g/L, 300 g/L and 300 g/L of TM40, HS30 and SM30, respectively, were performed with distilled water. Each stock solution was then diluted with distilled water in order to obtain a serial dilution to which the seeds of the common onion species *Allium cepa* were exposed to.

2.2.2. Characterization of NP suspensions

To characterize the NP suspensions dynamic light scattering (DLS) were used to measure the hydrodynamic diameter of particles in suspension (NP size), aggregation index and the polydispersity index (PDI) and electrophoretical light scattering (ELS) were used to measure the zeta potential of NP (measure of stability and surface charge) (HASSELLÖV et al., 2008; MALVERN INSTRUMENTS, 2008; KATO et al., 2009; SAYES; WARHEIT 2009; CARD; MAGNUSON, 2010). The zeta potential and the polydispersity index provided information about the stability and agglomeration of NP on the medium used for all tests. Also the DLS results provided information about the aggregation process of each particle. ASTM classification (1985) for the stability behavior of particles (i.e., to classify the zeta potential as a high or low value) was used to categorize the stability of the NP suspensions. Measurements were made in a Malvern Instrument Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK) at 20ºC and in backward scattering at 173º.

2.2.3. Data analysis

All data were analyzed using statistical analysis Kruskal-Wallis non-parametric test to determine treatment effects, and then to compare each treatment to the control using the software SAS 9.1 for Windows. All statistical analyses were based on 0.05 significance level.

2.3. *Allium cepa* assay

The *A. cepa* assay was performed according to a modified version of Grant's protocol (GRANT, 1982). All assays were carried out with only one kind of seed of *A. cepa* (variety Baia Periforme), to avoid different responses in the several stages of the process.

The *A. cepa* seeds were continuously exposed to different concentrations of SiNP (TM40 – 24.43 to 3.2 g/L, with a dilution factor of 1.5 x; HS30 and SM30 – 18.22 to 2.4 g/L with a dilution factor of 1.5 x), at 20 ± 5 ºC and 12:12 h light:dark, in Petri dishes covered with filter paper embedded with the NP solution, each with a different concentration. Four replicates per treatment, each one with 100 seeds were used. Control were carried out with distilled water (negative control) (LEME; MARIN-MORALES, 2008). The seeds were exposed to NP-suspensions until the roots from the negative control reached two cm length (which took 4 to 5 days). At this stage, the germination index, in %, was calculated as the proportion of the seeds with visible radicle protusion, afterwards 30 randomly picked roots from each replicate of each treatment were measured and then they were collected (only those with two cm) and fixed in Carnoy 3:1 (ethanol: acetic acid, v/v) for 6-12 h, and, afterwards, were transferred to a new Carnoy's fixative and stored at 4°C before further analysis.

The slide preparation with the meristematic root cells followed the procedure described by Leme and Marin-Morales (2008). The fixed roots were washed with distilled water and then hydrolyzed in HCl 1 N, in a bath at 60ºC for 8-11 min, afterwards they were washed in distilled water again and then stained with Schiff's reagent for two hours in the dark, then washed one more time with distilled water. The slides were prepared using the meristematic regions, they were cut onto the slide into a drop of 2% acetic carmine solution, to increase the cytoplasm contrast, and then covered with a cover slip and carefully squashed. Afterwards the cover slip was removed with liquid nitrogen and the slides were mounted in synthetic resin (Enthellan, Merck) to further analysis. In order to evaluate cell damages, 10 slides per

treatment were prepared and 500 intact cells were analyzed per slide, under an optical microscope (1000x), totalizing 5000 cells per test in each treatment.

Genotoxic potential was determined according to the observation and quantification of any chromosomal and nuclear abnormality in the meristematic cells, in all phases of the cell cycle, of all treatments, such as abnormal anaphases (e.g. multipolar, with bridges, delayed), fragments and loss of chromosomes, C-metaphases. The evaluation of mutagenic effects was carried out by scoring micronucleated cells of meristematic regions in all slides of all treatments. We also analyzed the mitotic index (MI), i.e. the number of cells in division. The MI was calculated as the ratio between the number of mitotic cells and the total number of cells scored and expressed as percentage.

2.4. Results

Through the characterization we could evaluate the stability and agglomeration of all SiNP on the culture media used for the tests. All the NP was influenced by the medium used (Table 1). The stability measured by the zeta potential was different for the particles, the NP TM40 were more stable. The diameter of the particles increased, the TM40 NP which has a size of 22 nm, had an average size of 37.2 nm when diluted. For HS30 (12 nm) the average size was 19.1 nm, and for SM30 the average size was 17.1 nm.

Table 1 – Results of the NP characterization using dynamic light scattering and electrophoretical light scattering

Medium	SiNP	Conc (g/L)	Diameter (nm)	PI	Zeta Potential (mV)	Nanoparticles
H2O dest.	TM40	0,01	37,2	0,122	-25,99	TM40
H2O dest	HS30	0,01	19,1	0,251	-0,38	HS30
H2O dest	SM30	0,01	17,1	0,262	-1,65	SM30

Conc=concentration; PI = polydispersity index; TM40: 22 nm; HS30: 12 nm; SM30 7 nm.

The effect of the silica nanoparticles suspension on the cell division and chromosome behavior of *A. cepa* is presented in Table 2. With increasing the concentration of the NP a decrease in the mitotic index was noticed ($p<0.05$, Kruskal-Wallis test). For all nanoparticles

and on all concentrations (except for TM40 and 5.4 g/L for HS30) cytological effects including the chromosomal aberrations and micronucleus, were observed in treated cells.

Table 2 – Distribution and cytological effects in *Allium cepa* root tip after treatment with different concentrations of three different silica nanoparticles dispersion (TM 40 – 22 nm; HS30 – 12 nm; SM30 – 7 nm)

NP	Concentration (g/L)	Interphase	Prophase	Metaphase	Anaphase	Telophase	MN	AB	MI (%)
Control	0	4170	634	103	37	56	0	0	16.6
TM40	24.43	4950	30	9	0	11	49*	0	1*
	16.2	4422	179	116	74	54	19*	26*	8.38*
	10.8	4730	141	63	32	34	26*	20*	5.4*
	7.2	4658	298	79	46	44	2	11*	9.34*
	4.8	4434	348	98	64	56	5	6*	11.32*
	3.2	4209	565	110	53	63	4	4	15.82
HS30	18.22	4955	37	4	3	9*	32*	0	1.06*
	12.05	4834	69	51	25	30	37*	9*	3.49*
	8.1	4762	125	54	28	31	12*	10*	4.76*
	5.4	4855	296	61	43	45	7*	10*	8.9*
	3.6	4375	400	118	51	56	4	6*	12.5*
	2.4	4229	600	76	43	52	2	3	15.42
SM30	18.22	4945	44	6	4	11	51*	0	1.3*
	12.05	4554	242	91	54	54	33*	17*	8.79*
	8.1	4699	158	60	45	38	24*	24*	6.02*
	5.4	4837	77	39	24	23	12*	12*	3.26*
	3.6	4283	483	110	55	69	0	9*	14.34
	2.4	4056	743	95	45	61	3	5	18.88

MN: Micronucleos, AB: Chromosomal aberration and MI: Mitotic index. Asterisks indicate significant differences relatively to the negative control (* $p < 0.05$ Kruskal wallis test)

Phytotoxic effects were also observed. The germination rate presented significant values ($p<0,05$, Kruskal wallis) for all particles and for all concentrations when compared with control, except for SM30 2.4 g/L (Figure 1). All nanoparticles also reduced root growth, TM40 and HS30 has significant values for all concentrations and SM30 only on 8.1, 12.05 and 18.22 g/L. HS30 was the particle that caused more effect on root growth (Figure 2).

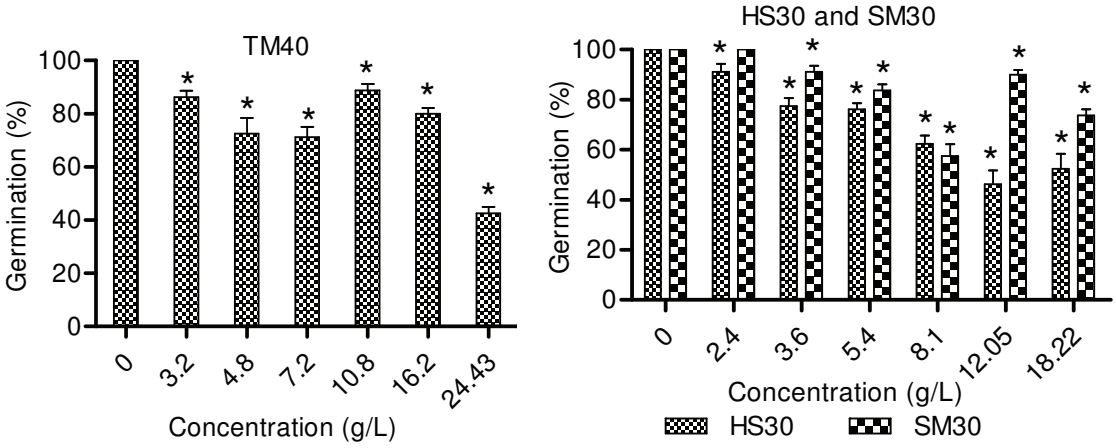

Figure 1 – Mean and SD of *Allium cepa* germination rate (in percentage) after exposure to three silica nanoparticles (TM40 – 22 nm; HS30 – 12 nm and SM20 – 7nm) with different concentrations. Asterisks indicate significant differences relatively to the negative control (* $p < 0.05$, Kruskal wallis test)

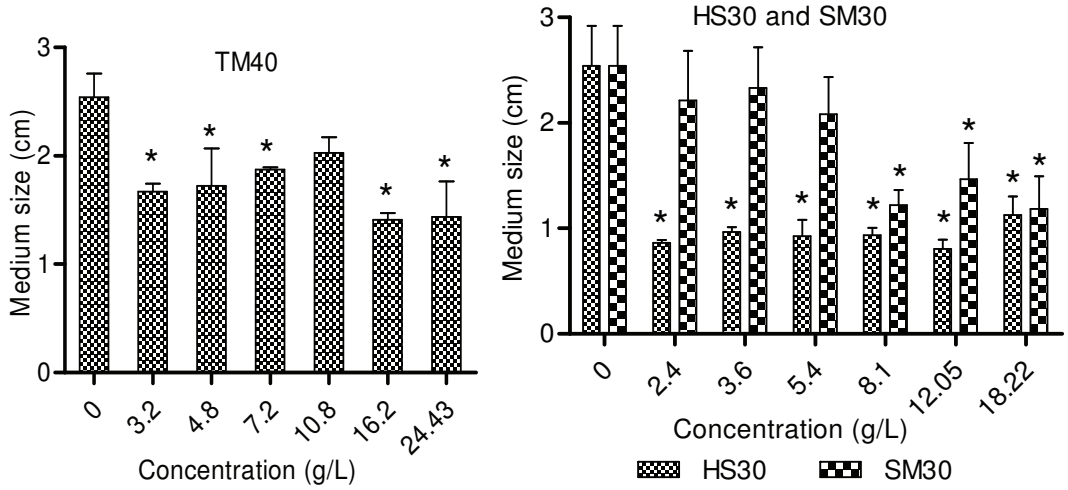

Figure 2 – Mean and SD of *Allium cepa* root length (in cm) after exposure to three silica nanoparticles (TM40 – 22 nm; HS30 – 12 nm and SM20 – 7nm) with different concentrations. Asterisks indicate significant differences relatively to the negative control (* $p < 0.05$ Kruskal wallis test)

2.5. Discussion

The SiNP exhibited cytotoxicity by decreasing the mitotic index in a dose-dependent manner. The cytotoxicity level of a test compound can be determined based on the increase or decrease in the mitotic index (MI), which can be used as a parameter of cytotoxicity in studies of environmental biomonitoring (FERNANDES et al., 2007) Therefore SiNP may have a

mito-depressive effect on the *A. cepa*. Other studies with nanoparticles have already shown that these chemicals can decrease mitotic index and cause mito-depressive effects on the *A. cepa* cells (KUMARI et al., 2009; 2011).

Different kinds of chromosomal aberrations were observed with different concentrations of the nanoparticle suspensions, such as chromosomal breaks, bridges, stickiness, disturber metaphase and micronucleus (Figura 3). Studies with mammalian cell lines also demonstrated that the nanoparticles penetrated subcellular structures increasing oxidative stress (PARK; PARK, 2009; WANG et al., 2009; PASSAGNE et al., 2012; AHAMED, 2013), and also its toxicity was dose- and size- dependent (KYUNG et al., 2009; NAPIERSKA et al., 2009). These papers suggest that amorphous SiNP below 100 nm induced cytotoxicity, so the size of the particles is critical to produce biological effects.

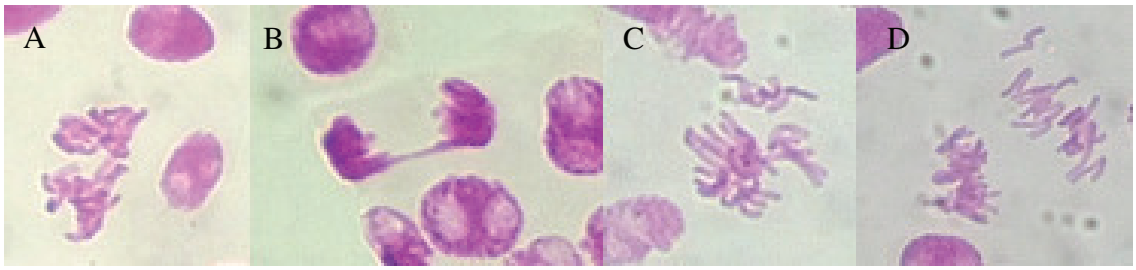

Figure 3 – Silica nanoparticles induced chromosomal aberrations in root tip cells of *A. cepa*. A: stickiness; B: chromatin bridge; C and D: multiple chromosomal breaks. Magnification 40x

According to Brunner et al. (2006) the toxicity of nanoparticles may be attributed duo to two actions, the first is based on the chemical composition, e.g., release of (toxic) ions, and the second is based on stress or stimuli caused by the surface, size and/or shape of the particles. In this case we are attributing the caused of the toxicity to the size of the NP, but this may not be the only factor.

Phytotoxic effects were observed to all SiNP studied, and also for growth and for germination effect were dose- and size-dependent. These data were also observed by Slomberg and Schoenfisch (2012), they studied phytotoxicity of SiNP (i.e., 14, 50 and 200 nm) for *A. thaliana* and found that SiNP can reduce plant development, also their results show that SiNP accumulates in *A. thaliana* root cells in a size-dependent manner. Stampoulis, Sinha and White (2009) studied germination and root growth of *C. pepo* treated with SiNP (<100 nm), and it inhibited completely germination at 1000 mg/L and have significant impact on root elongation.

2.6. Conclusions

In conclusion SiNP can penetrate the plant system causing effects on growth, germinations and on cell division. Plants are an important component of the ecosystems so those findings suggest that they need to be included when evaluating the toxicological impact of the nanoparticles on the environment.

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3. TOXICITY AND GENOTOXICITY OF DIFFERENT SIZES SILICA NANOPARTICLES IN *DANIO RERIO*

Abstract

Nanotechnology has been increasing rapidly but their effects on human health and on the environment are still poorly known. Silica nanoparticles (SiNP) are been used extensively on different areas, from drug delivery, printer toners to cosmetics and food. This study aims to investigate whether the size of SiNP caused toxicity and/or genotoxicity during *Danio rerio* embryogenesis. To this purpose, three different sizes of SiNP (TM40 – 22 nm, HS30 – 12 nm and SM30 – 7 nm) were used and early life-stages assay and comet assay with *Danio rerio* were applied. The measure endpoints for the early life-stages were edemas, body malformation, tail malformation, hatch, mortality and growth. At the end of the embryo test (96 h) a significant increase of edema and mortality was found when increasing concentration for all particles. For deformation, significant data was only found for the HS30 in 6 g/L concentration. By comparing the effective concentrations causing 20 and 50% of effect (EC20 and EC50) it is notice that the intermediate size particle caused more effects than the others, followed by the smaller size particle. The particle that caused less effect was the greater particle. For the comet test, the HS30 was the particle that caused more effect than the others. For TM40 and SM30, intermediate concentrations caused more effects. These results show that the exposure to SiNP can cause not only morphologic effects on *Danio rerio*, but also genotoxic effects. Also the size of the particles and the physical and chemical characteristics can strongly influence the toxicity.

Keywords: Nanotoxicology. Comet assay. Embryonic assay.

3.1. Introduction

With the rapidly increase of nanotechnology it is necessary also to increase the studies about its effects not only on human health but also on environmental health. Due to this rapidly increase of nanoparticles (NP) and nanotechnology these products are likely to result in releases into the environment; therefore concerns about the potential environmental contamination have been arisen. Silica nanoparticles (SiNP) have been highly studied, and extensive applications for these type of particles have been found, it can be used from drugs delivery (SLOWING et al., 2008), printer toners (WINNIK et al., 1992), cosmetics (FERRARI; KHACHIKIAN, 2006) to food (DEKKERS et al., 2011). The potential that these types of NP have to contaminate the environment depends on a large number of factors, such as size, surface/volume ratio, shape, chemical composition, etc., and all these factors can be influenced by environmental parameters, therefore investigation into their ecotoxicological potential need to be further studied. Although many studies have shown cytotoxicity of SiNP *in vitro* and *in vivo* (NAPIERSKA et al., 2009; YE et al., 2010; LU et al., 2011; PASSAGNE et al., 2012; AHMAD et al., 2012) just a few (VAN HOECKE et al., 2008; BING et al., 2009; FENT et al., 2010; SAFEKORDI et al., 2012; CASADO et al., 2013) used aquatic animals, and since the aquatic environment is the ecosystem that is most harmed by contaminants, studies with aquatic species are very important to evaluate the SiNP effects on this habitat.

Danio rerio has been used for many NP studies (ZHU et al., 2008; USENKO et al., 2007; LEE et al., 2007; CHENG et al., 2007) and it is an attractive model system to study effects of NP environmental exposure (ISPAS et al., 2009). The development and cellular composition of major organ systems of *Danio rerio* allows it to be used as a test organism to calculate effects of nanoparticles release (SCHOENEBECK; YELON, 2007; DRUMMOND, 2005). Also the similarities in the organ physiology between *D. rerio* and other vertebrates make it a good model system to identify potential health effects in humans (ISPAS, 2009).

Zebrafish (*D. rerio*) is an attractive model for rapid screening of developmental and toxicological effects from NP exposure due to a series of characteristics. For example, during embryogenesis, they have a small size, their development is fast, they are easy to take care, we can easily obtain large numbers of individuals and have good optical clarity (WESTERFIELD, 2000). Also it has been shown to be particularly sensitive to pollutants, such as NP, interacting with DNA (ZHU et al., 2008; GRIFFITT et al., 2009; CHEN et al., 2011; OSTERAUER et al., 2011; XIONG et al., 2011; ZHAO et al., 2013).

Genotoxicity is a relevant factor for aquatic organisms, and it may provide a basis for an ecotoxicologic risk assessment of genotoxic substances, that may enter the aquatic environment (DIEKMANN et al., 2004). Substances that caused genotoxic effects can cause several consequences on organisms, such as gamete loss, decrease in reproductive success, abnormal development, cancer, lethal mutations, embryo mortality, as well as increase or decrease genetic diversity (WÜRGLER; KRAMERS, 1992; ANDERSON; WILD, 1994)

For this type of study the comet assay is well recommended because it is a simple and sensitive technique, measuring and analyzing DNA damage in individual cells (MCKELVEY-MARTIN et al., 1993; COLLINS et al., 1997). The comet assay has been applied repeatedly to environmental monitoring studies (MCKELVEY-MARTIN et al., 1993; FAIRBAIRN et al., 1995; MITCHELMORE; CHIPMAN, 1998; COTELLE; FERARD, 1999; LEE; STEINERT, 2003; KOSMEHL et al., 2004) and also on NP studies (BARNES et al., 2008; KARLSSON et al., 2008; REEVES et al., 2008; GOPALAN et al., 2009; FLOWER et al., 2012; DE LIMA et al., 2013).

The objective of this study was to determine whether the size of SiNP alters the level of toxicity and genotoxicity during zebrafish embryogenesis, using three different sizes of SiNP (TM40 – 22 nm, HS30 – 12 nm and SM30 – 7 nm) to identify toxicity levels.

3.2. Methodology

3.2.1. Characterization of NP suspensions

For this study, was used SiNP, electrostatically stabilized with negative charge, sodium as counterion and with different sizes: TM40 (size: 22 nm), HS30 (size: 12 nm) and SM30 (size: 7 nm) (supplied by the Chemical Departament of the Coimbra University) Stock suspensions of 400 g/L, 300 g/L and 300 g/L of TM40, HS30 and SM30, respectively, were performed with distilled water.

To characterize the NP suspensions dynamic light scattering (DLS) were used to measure the hydrodynamic diameter of particles in suspension (NP size), aggregation index and the polydispersity index (PDI) and electrophoretical light scattering (ELS) were used to measure the zeta potential of NP (measure of stability and surface charge) (HASSELLÖV et al., 2008; MALVERN INSTRUMENTS 2008; KATO et al., 2009; SAYES; WARHEIT 2009; CARD; MAGNUSON, 2010). The zeta potential and the polydispersity index provided

information about the stability and agglomeration of NP on the medium used for all tests. Also the DLS results provided information about the aggregation process of each particle. ASTM classification (1985) for the stability behavior of particles (i.e., to classify the zeta potential as a high or low value) was used to categorize the stability of the NP suspensions. Measurements were made in a Malvern Instrument Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK) at 20ºC and in backward scattering at 173º.

3.2.2. *Danio rerio* early life-stages assay

The *Danio rerio* early life-stages assay was based on the OECD draft guideline on Fish Embryo Toxicity (FET) Test (OECD, 2006ª). The test started by exposing newly fertilized eggs of *Danio rerio* to the three SiNP in a concentration range from 9 to 1.77 g/L (HS30), 12.15 to 2.4 g/L (SM30) and 60.8 to 12 g/L (TM40). Thirty eggs per treatment (3 replicates) were selected and distributed in 24-well microplates. Exposure occurred for 96h at 28±1ºC and a photoperiod of 16:8 h light:dark. Embryos and larvae were daily observed under a stereomicroscope (Stereoscopic ZoomMicroscope-SMZ 1500, Nikon Corporation, Japan) magnification used for observations was 70x for eggs and 40x for larvae. The following parameters were evaluated: edemas, body malformation, tail malformation, hatch, mortality and growth.

3.2.3. Comet assay

For the Comet assay, 10 eggs were exposed, for 48h, to the same range of concentrations as the early life-stages assay, in order to evaluate effects in DNA integrity (comet assay). The comet assay detects a variety of DNA lesions including SSB (Single Strands Breaks), alkali-labile sites, cross-linking lesions, and excision repairs SSB. The cell isolation was carried out mechanically according to the protocol by Kosmehl et al. 2006. The comet assay (embedding, lysis and electrophoresis) was carried out under alkaline conditions. All slides were examined at 340x magnification using a fluorescence microscope. One hundred cells per slide were scored, and 5 slides were analyzed for each concentration of nanoparticles.

3.2.4. Statistical analysis

Statistical analyses were carried out with SAS 9.1 for Windows. Multifactor variance analyses (ANOVA) were carried out to detect the significant differences between the groups for normally distributed data sets. In case of significant values the Tukey's post hoc test were conducted. When data did not fit the assumptions of normality, Kruskal-Wallis non-parametric tests were used to determine treatment effects, and then to compare each treatment to the control. EC50 and EC20 were calculated with the PriProbit software package (SAKUMA, 1998). All statistical analyses based on 0.05 significance level.

3.3. Results

3.3.1. Nanoparticles Characterization

Through the characterization of the NP we could evaluated the stability and agglomeration of all SiNP on the culture media used for the tests. The zeta potential and the polydispersity index provide information about the stability and agglomeration of NP on the medium used for all tests. Also the DLS results provide information about the aggregation process on each culture media. All the NP was influenced by the culture media used (Table 1). SM30 was the particle that had a greater change, the diameter change from 7 nm to 18 nm. HS30 was the particle that presented lower polydispersivity index (0.13) and highter zeta potential (-24.9). The polydispersivity index for TM40 was 0.225 and the zeta potential it was -53.32, for SM30 was 0.251 and -48.87 respectively.

Table 1 – Results of the NP characterization using dynamic light scattering and electrophoretical light scattering

Medium	SiNP	Concentration (g/L)	Diameter (nm)	polydispersity index	Zeta Potential (mV)	Notes
H2Osyst	TM40	15.2	26.4	0.225	-53.32	TM40-22nm
H2Osyst	HS30	3	17.8	0.13	-24.9	HS30-12nm
H2Osyst	SM30	4.05	18	0.251	-48.87	SM30-7nm

3.3.2. *Danio rerio* early life-stages assay

In the fish embryotocixity test using NP as a contaminant, fertilized zebrafish embryos were exposed for a period of 96 h. The results are presented as stacked bars in the Figure 1. An increase of edema and deformation was observed with increasing time, also the embryos exposed to the NP hatched earlier than the control, this for all particles and all concentrations (Figure 1).

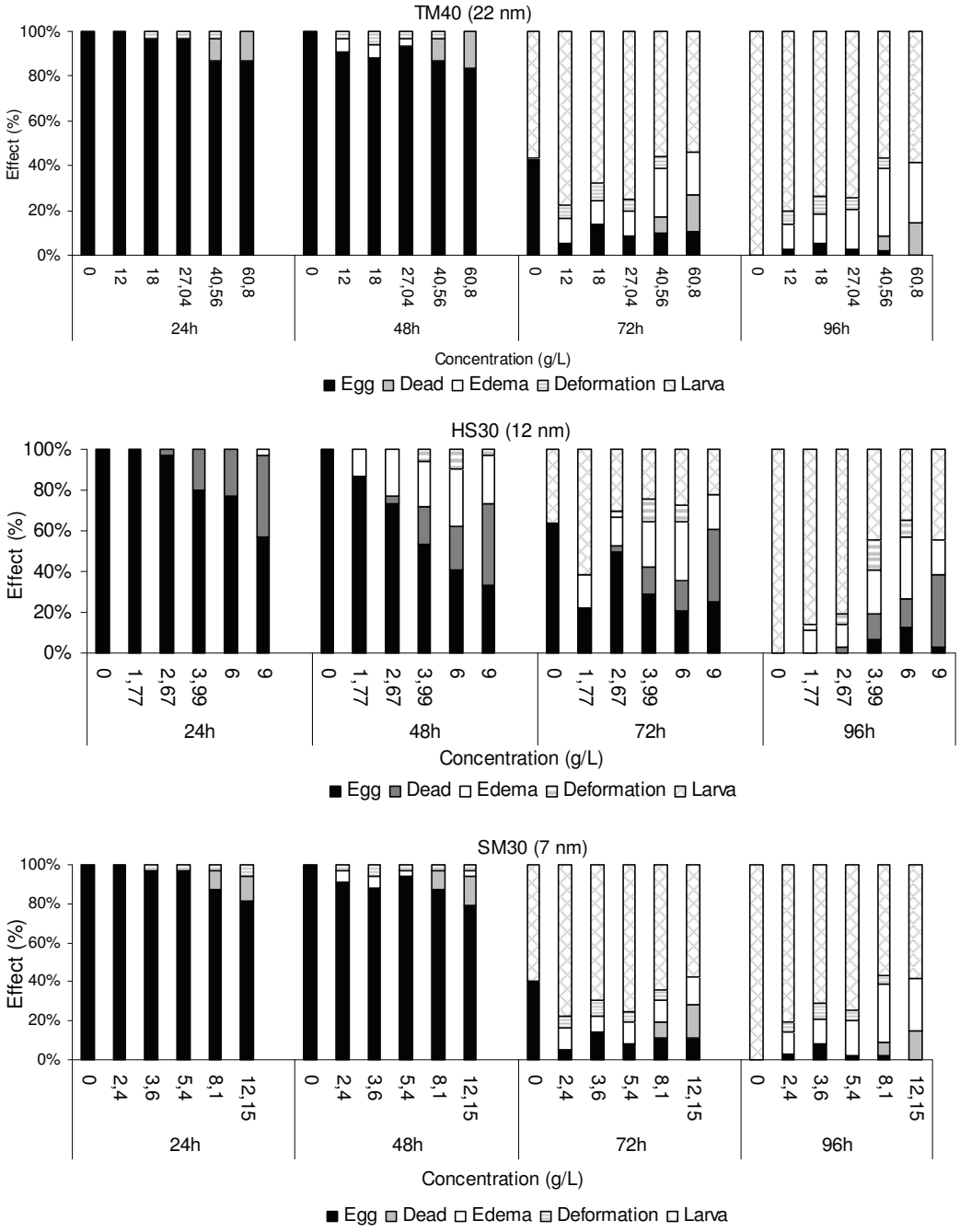

Figure 1 – General overview of silica nanoparticles effects on *D. rerio* embryo and larvae during 96 hours of exposure.

The different photographs in Figure 2 indicate the occurrence of deformations and edema, it is possible to distinguish between different types of deformations.

Figure 2 - Effects observed on *D. rerio* under stereomicroscopyon (Magnification 40x) after 96 hours of exposure: Edema (a); edema and deformation (b c, and d); no hatching (c)

At the end of the embryo test (96 h) a significant increase of edema and mortality was found in fish when increasing concentration for all particles. For deformation significant data was only found for the HS30 with 6 g/L concentration. The particles TM40 and HS30 also presented significant values for hatch, concentrations 60.8 g/l for TM and 6 g/L for HS.

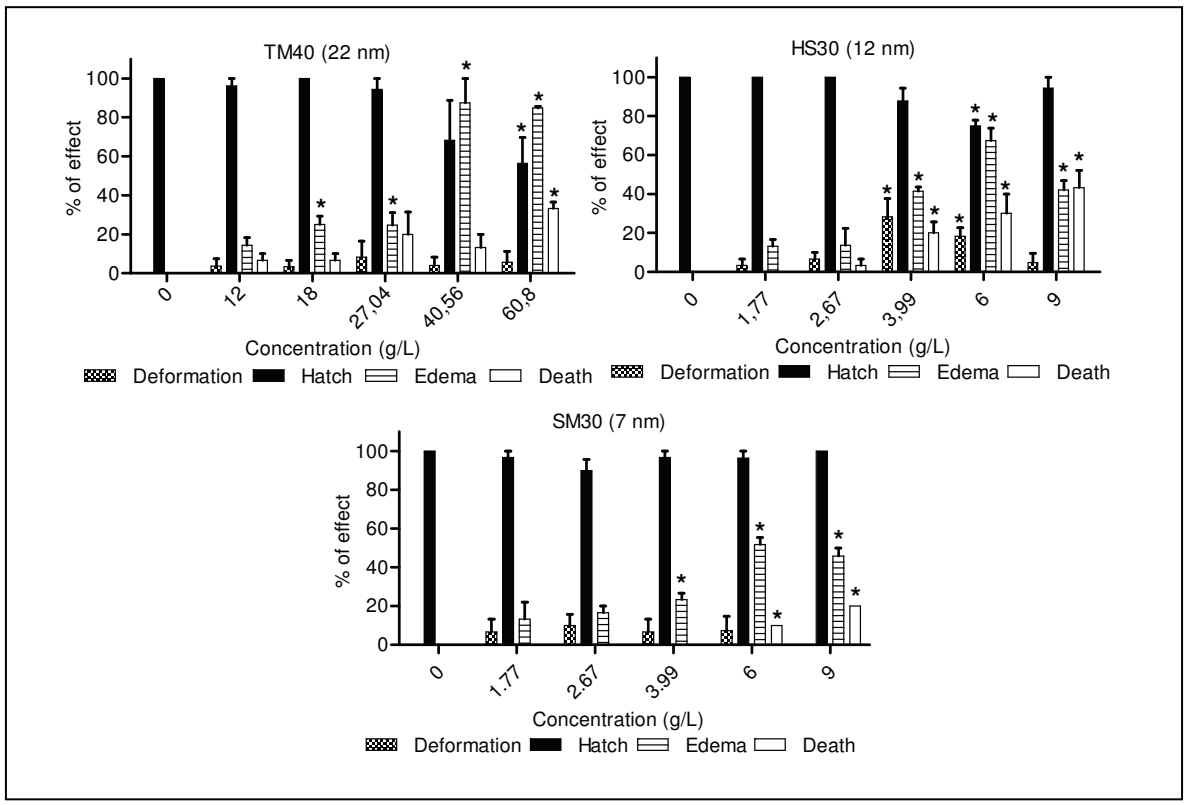

Figura 3 – Percentage of dead, deformation, edema and hatch *D. rerio* embryo and larvae after 96 hours of exposure. *=P<0.05 (Kruskal-Wallis test)

With increasing concentration of the nanoparticles, a decrease of the growth was noticed. Significant values were observed on 40.56 and 60.8 g/L for TM40, 3.99, 6 and 9 g/L for HS30 and for SM30.

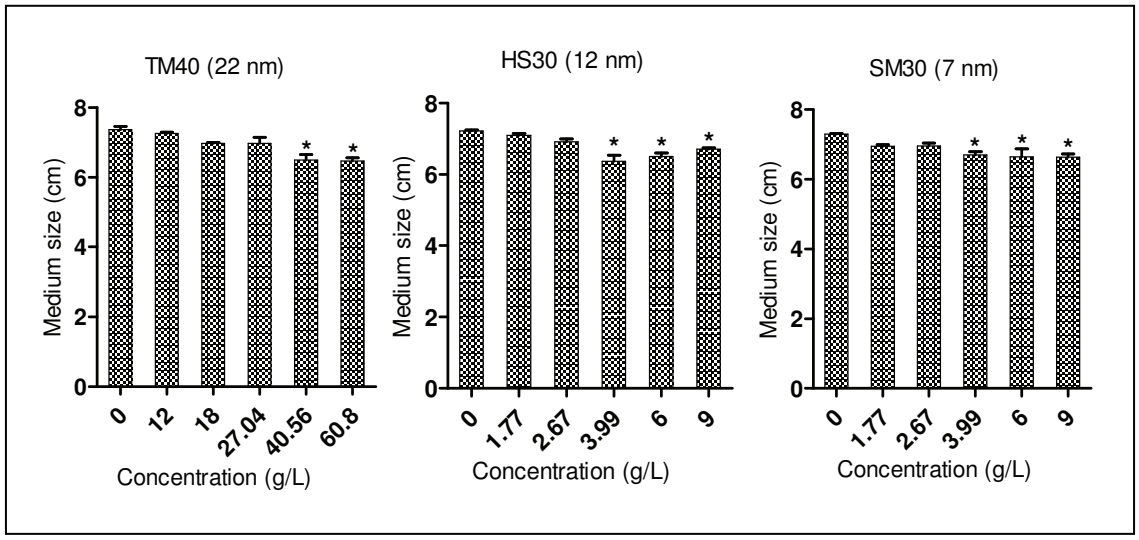

Figure 4 – Medium size of D. rerio after 96 hours of exposure to several concentrations of SiNP. *=P<0.05 (Tukey test)

By comparing the effective concentrations causing 20 and 50% of effect it is notice that the intermediate size particle (HS30) caused more effects than the others, followed by the smaller size particle (SM30). The one that caused less effect was the greater particle (TM40) (Table 1).

Table 2 - Effective concentration (g/L) causing 20 and 50% of effect after exposure of the fish *Danio rerio* to the three nanoparticles (NP)

NP-SiO2	Size	Endpoint	72 h
TM40	22nm	EC_{20}	3,45g/L (2,58-4,15)
		EC_{50}	5,85g/L (5,00-6,78)
HS30	12nm	EC_{20}	0,61g/L (0,35-0,73)
		EC_{50}	1,13g/L (0,91-1,35)
SM30	7nm	EC_{20}	0,81g/L (0,35-1,23)
		EC_{50}	2,68g/L (2,01-4,53)

3.3.3. Comet assay

Different sizes of SiNP were tested to assess the possibility of size-dependent genotoxicity. Although smaller nanoparticles may be expected to be more reactive and

therefore potentially more toxic, there were more significant results for the HS30, particle with intermediate size, when compared with the control, in this case it was observed significant results for all concentrations tested (Figure 5). For TM40 and SM30 intermediate concentrations caused more effects (Figure 5).

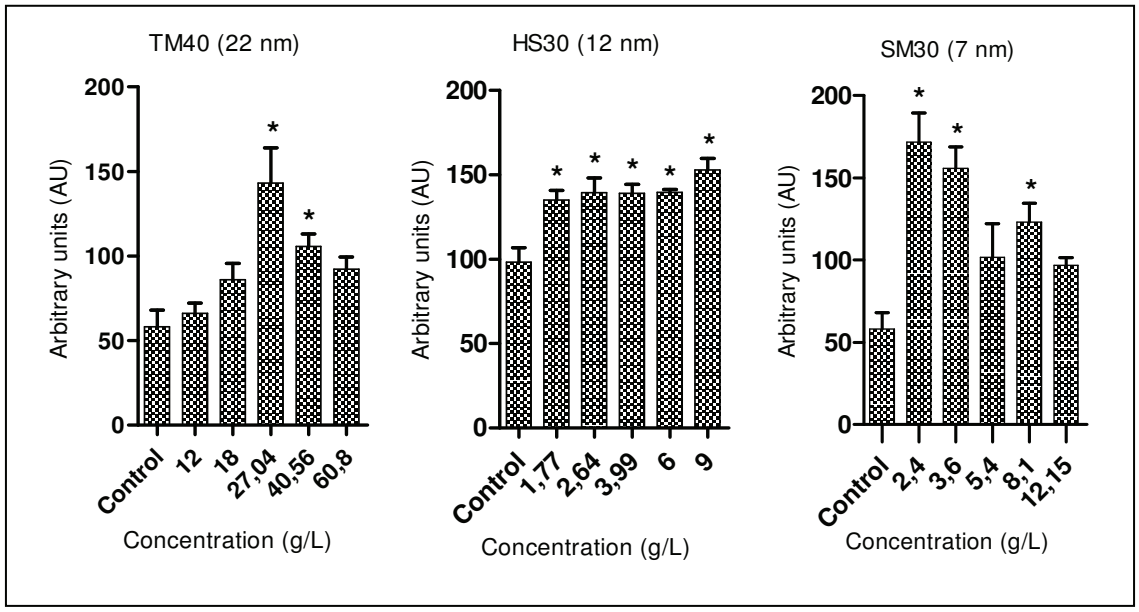

Figure 5 – Effect of sílica nanoparticles (TM40, HS30 and SM30) with different concentrations, after 48 hours exposure. *=P<0.05 (Tukeys test).

3.4. Discussion

The *Danio rerio* species has similar physiological reactions to those observed in mammals, rapid embryonic development and an optical transparency of their embryos, and for these advantages they are being highly indicated for study the effects of NP. From the results it can be implied that the NP size has a great importance on toxicity, the results show that the greater particle (TM40 – 22 nm) have less effects than the others. The intermediate particle (HS30 – 12 nm) has the greater effect, this can be explained by the chemical analyses, this particle suffer less influence by the medium then the others. The SM30, that was supposed to cause more effect, didn't. Through the chemical analyzes it could be observed that the size of this particle was drastically changed, from 7 nm to 18 nm, this happened due to the lack of stability and higher aggregation of this particle on the medium use. Fent et al. (2010) tested SiNP (fluorescent core-shell – 60 and 200 nm) on *D. rerio*, for 96h and did not observe effects on hatching time and hatching success, mortality or deformities, but in his studies the SiNP were much larger than in this presented study, strengthening the assumption that size matters when it comes to nanoparticles toxic effects.

Through the comet assay we could observe that, differently from the early life stage assay, it was observed larger effects on the intermediate concentration for TM40 and SM30. But, like in the early life stage assay, the particle HS30 was the one that caused more effect, followed by the SM30. In water NP attempt to aggregate, there for these results can be explained by the size change, cause by these characteristic, which may hamper the entrance of NP into cells. Also the particle concentration may effect the size distribution of NP, therefore the effects on the intermediate concentrations. Barnes et al. (2008) studies the genotoxicity of amorphous SiNP in the size range from 20 nm to below 400 nm and does not detect any genotoxicity in his comet assay. Kim et al. (2010) also tests SiNP (LUDOX AS-20, 16.9 nm; LUDOX AM, 15.3 nm and LUDOX CL, 13.3 nm), and differently from Barnes, all of the nanoparticles of these study have shown some genotoxicity, in the same way that this paper shows.

3.5. Conclusion

In brief, the present study demonstrates that exposure to silica nanoparticles can cause not only morphologic effects on *Danio rerio*, but also genotoxic effects. Also the size of the particle and the physical and chemical characteristics can strongly influence on the toxicity of the particle.

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4. TOXICITY OF THREE SILICA NANOPARTICLES TO THE BACTERIA *VIBRIO FISCHERI* AND THE ALGAE *RAPHIDOCELIS SUBCAPTATA*

Abstract

Recently nanoparticles (NP) have received a lot of attention and generate great amount of concern due to their rapidly increasing applications in many areas. There are numerous applications for silica nanoparticles (SiNP) and it can be used in many fields: chemical industries, biomarkers in medicine, cosmetics, paints, food and medicine. Not much is known about how these particles act on the aquatic environment. To evaluate size-dependent toxicity of silica nanoparticles (TM40 – 22nm, HS30 – 12nm and SM30 – 7nm), the marine bacteria *Vibrio fischeri* and the freshwater algae *Raphidocelis subcapitata* tests were used. SiNP have shown toxic effects for both organisms, and the bigger particle were less toxic than smaller one, demonstrating that silica nanoparticles toxicity is size-dependent and can cause harm to the marine and freshwater aquatic environment.

Keywords: Nanotoxicology. Nanoparticles. Bioluminescence. Growth rate. Size-dependence.

4.1. Introduction

Recently nanoparticles (NP) have received a lot of attention and generate great amount of concern duo to their rapidly increasing applications in many areas, such as textiles, electronics, pharmaceutics, cosmetics, environmental remediation and others (NAVARRO et al., 2008; DUNPHY GUZMAN et al., 2006; THE ROYAL SOCIETY, 2004). Silicate is a common substance in the environment and it causes no harm in its environmental form, but when transform into NP, its characteristics may change, so toxicity studies are very important to determinate whether its use is safe, not only the population but also for the environment. There are numerous applications for silica nanoparticles (SiNP) and it can be used in many fields: chemical industries, biomarkers in medicine, cosmetics, paints, food and medicine (WEI et al., 2010; FEDE et al., 2012.) . Several studies have shown that these particles can cause damage to human cells (LU et al., 2011; OGS; CHOI, 2009; SUN et al., 2011; PASSAGNE et al., 2012). Much of what we know about NP toxicity has been derived from biomedical investigations (HOWARD, 2004; THE ROYAL SOCIETY, 2004), not much is known about how these particles act on the aquatic environment.

All types of products tend to end up somehow in waterways (e.g., rivers, lakes, estuaries, etc.) (DAUGHTON, 2004; MOORE, 2002; MOORE et al., 2004). So as the nanotechnology grow it is inevitable that NP and nanoscale products and by-products enter the aquatic environment (DAUGHTON, 2004; HOWARD, 2004; MOORE, 2002; MOORE et al., 2004; THE ROYAL SOCIETY, 2004). So it is extremely important to evaluate potential hazards and to take safety measures, so that the environment can be protected.

One of the widely used tests in aquatic ecotoxicology is the *Vibrio fischeri* luminescence inhibition assay, an international recognized and standardize test as ISO (2007) (LIBRALATO et al., 2010). Though the microtox ® is a widespread acute test, it is very sensitive, reproducible, possesses high discriminant power for organic and inorganic pollutants (CZECH; OLESZCZUK, 2014), and is also quite well predicting the toxicity of chemicals to other in vitro systems (KAHRU, 2006; MORTIMER, 2008). This test uses the bioluminescence of the marine bacteria *Vibrio fischeri* as an indicator of toxicity.

Other widely used assay is the algae growth inhibition assay (BLINOVA, 2004). The algae *Raphidocelis subcapitata* (Former known as *Pseudokirchneriella subcapitata*) is considered a model organism for freshwater algae and is widely used in the OECD algal growth inhibition assay (ORGANISATION FOR ECONOMIC CO-OPERATION AND

DEVELOPMENT, 1994) as well as in US EPA green algae growth inhibition test (USEPA, 1996).

Both organisms, *V. fischeri* and *R. subcapitata*, were successfully used to evaluate NP toxicity on aquatic environment (ARUOJA et al., 2009; FRANKLIN et al., 2007; VAN HOECKE et al., 2008; HEINLAAN at al., 2008).

This paper aims to evaluate size-dependent toxicity of three silica nanoparticles (TM40 – 22nm, HS30 – 12nm and SM30 – 7nm) on the aquatic environment, by using marine bacteria *Vibrio fischeri* and the freshwater algae *Raphidocelis subcapitata*.

4.2. Methodology

4.2.1. Studied NP

Silica nanoparticles, electrostatically stabilized with negative charges and with sodium as counterion and with different sizes were studied in the present work: TM40 (size: 22 nm), HS30 (size: 12 nm) and SM30 (size: 7 nm) (supplied by the chemistry department of the coimbra university). Stock suspensions of 400 g/L, 300 g/L and 300 g/L of TM40, HS30 and SM30, respectively, were performed with distilled water. Each stock solution were afterwards diluted with each organims culture media and the species test were exposed.

4.2.2. *Vibrio fisheri* bioluminescence inhibition assay

The ecotoxicity of the three SiNP was assessed for the marine bacteria *Vibrio fischeri* through carrying out the ASTM extended 9 dilutions test protocol (consisting of nine test dilutions) (AZUR ENVIRONMENTAL, 1998). The principle of this assay holds on the exposure of the bacteria to aqueous samples and measures the increase/decrease in the bacteria's light output (AZUR ENVIRONMENTAL, 1998). The highest test concentrations assayed for TM40, HS30 and SM30 were 163.8, 122.85 and 122.85 g/L respectively. All tests were performed using the Microtox 500 Analyzer and bioluminescence measurements were monitored at 0, 5, 15, and 30 minuts of exposure. The effective concentrations causing 20% and 50 % (toxic effects threshold), EC_{20} and EC_{50} respectively, of bioluminescence inhibition were computed for each NP by using the Software for MicrotoxOmni Azur (AZUR ENVIRONMENTAL, 1998). To account for possible interferences in the luminescence

measurements, absorbance was measured for each tested NP suspension, without the addition of the bacteria. For all NP suspensions measured absorbance was zero.

4.2.3. *Raphidocelis subcapitata* growth inhibition assay

Algal growth inhibition assay with the green microalga *R. subcapitata* (formerly *Raphidocelis subcaptata*) were conducted following the standard guideline of OECD (2006). Prior to the start of the assays, an inoculum was incubated in algal growth media (Woods Hole MBL medium Culture-MBL) for four days under control conditions (20±1ºC; photoperiod 16:8 h light:dark) to ensure that the microalgae were in exponential growth, as recommended by OECD (2006), at the beginning of the assay. The assays were performed in sterile 24-well microplates by carrying out three replicates per tested concentration and control (consisting of MBL). The microalga *R. subcapitata* were exposed for a 72 h period to a range of five concentrations of each SiNP using MBL as dilution water (TM40 – 6.25 to 0.19 g/L; HS30 and SM30 – 9.37 to 0.29 g/L) plus a control (MBL), at 24±1ºC and with a constant luminous intensity (60–120µE/m2/s, equivalent to 6,000–10,000 lux). Each well in the microplates was filled with 900 µL of test solution and inoculated with 100 µL of the correspondent algal-inoculum solution (10^5 cells/mL), so that the nominal initial cell concentration was 10^4 cells/mL. The peripheral wells, in the 24-well microplates, were filled with 500 µL of distilled water, to minimize evaporation in the assay wells. During the incubation period all vessels were shaken manually two times a day. After 72 h of exposure the concentration of algae was computed at each replicate by measuring absorbance at 440 nm (Jenway, 6505 uv/vis spectrophotometer) and using the equation: $C = -17108 + ABS \times 7925350$, where C is the algae concentration (cells per milliliter) and ABS is the absorbance obtained at 440 nm. For each concentration, the average specific growth rate (μ, d^{-1}) (for exponentially growing cultures) and the percentage reduction in average growth rate compared to the control value were calculated, after a period of 72-h.

The growth rate was calculated according to the following equation: $\mu=(\ln N_t-\ln N_{t0})/(t-t_0)$, in which μ is the growth rate, $t-t_0$ is the NP exposure period, and N_t and N_{t0} are the cell densities at time t and at the beginning of the test, respectively. And the percentage reduction was calculated according to the following equation: $R=[(C-\mu)/C]x100$, in which R is the percentage reduction, C is the control and μ is the growth rate.

In order to exclude any potential interference of some color that NP could present in suspension, the absorbance of each NP suspension without algae was also measured at 440 nm.

4.3. Results

4.3.1. Microtox assay

No inhibition of bioluminescence of *V. fischeri* was induced by the control (without the addition of NP). The EC20 and EC50 (threshold for effects) values after 5, 15 and 30 minutes of exposure and the corresponding 95% confidence interval were computed for each NP (Table 1). All the particles induced effects to the bacteria *V. fischeri* (Table 1). The toxicity of all NP increased with exposure time (Table 1). For a better perception of the observed responses the actual toxicity curves obtained after exposure of *V. fischeri* to the NP-suspensions (TM40, HS30 and SM30) are given in Figure 1.

Comparing the ratios EC50/EC20, for all the particles, the SM30 exhibited the highest toxicity followed by HS30 and TM40 (in decreasing order of toxicity) (Table 1). This suggests that toxicity increases, if the size of the SiNP decreases.

Table 1 – Effective concentration (g/L) causing 20 and 50% of effect after 5, 15 and 30 min of exposure of the bacterium *V. fischeri* (Microtox assay) to the three nanoparticles (NP)

NP	Endpoints	5 min	15 min	30 min	Trend
TM 40	EC50	29.11 (23.77 - 35.66)	19.03 (16.99 - 22.65)	14.07 (11.92 – 16.61)	↓
	EC20	6.28 (4.85 - 7.80)	4.94 (3.89 - 6.04)	3.81 (3.00 - 6.04)	↓
HS30	EC50	32.34 (25.70 - 40.71)	14.26 (11.79 - 17.24)	9.82 (8.39 - 11.48)	↓
	EC20	10.10 (8.26 - 12.02)	5.17 (3.20 - 7.32)	4.05 (2.55 - 5.68)	↓
SM30	EC50	4.58 (3.82 - 5.50)	2.77 (2.00 - 3.82)	2.45 (1.75 - 3.43)	→
	EC20	0.9 (0.60 - 1.23)	0.72 (0.51 - 0.96)	0.72 (0.51 - 0.94)	→

Arrows highlight the trend (↓ decrease, or → no change) of EC values along the exposure time. All EC50 and EC20 results were in g/L.

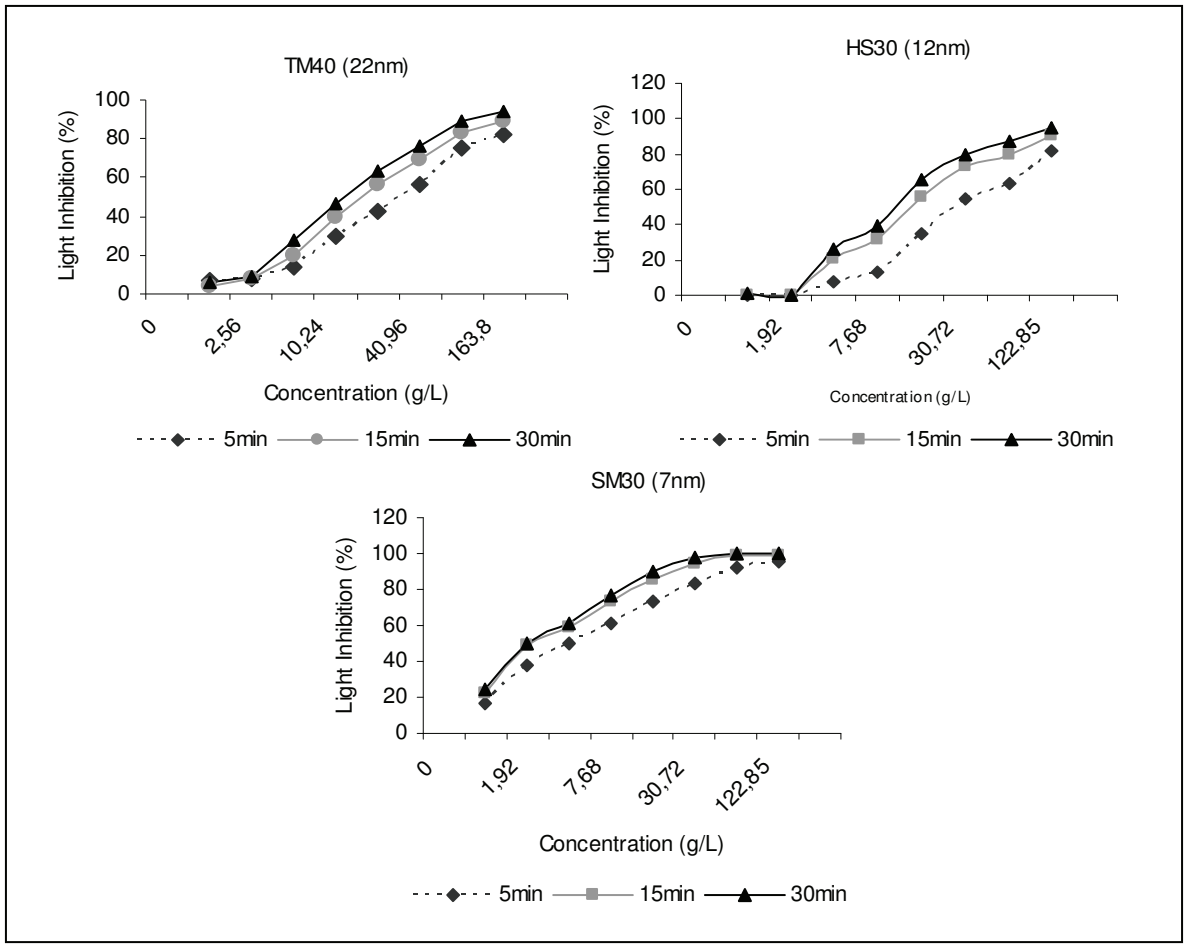

Figure 1 – Percentage of inhibition in bioluminescence of *V. fischeri* exposed, for 5, 15 and 30 min, to serial dilutions of suspensions NP.

4.3.2. *G*rowth inhibition assay with *R. subcapitata*

Concentration-response curves obtained on growth-inhibition experiments with SiNP (TM40, HS30, SM30) are shown in Figure 2. The nanoparticle suspensions clearly induced toxic effects to *R. subcapitata*.

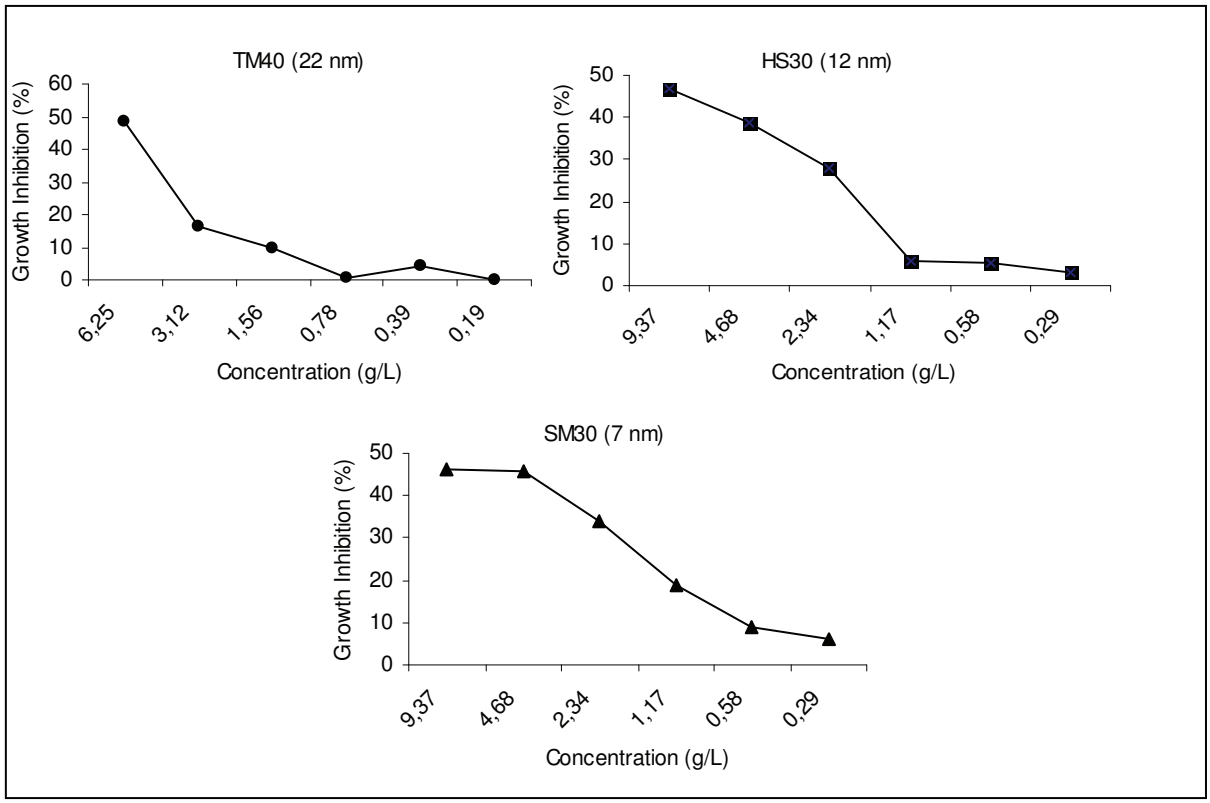

Figure 2 – Percentage of inhibition on growth of *R. subcapitata* exposed to a serial dilutions of nanoparticles suspensions

The EC20 and EC50 values derived from the experiments were used to compare the toxicity all particles (Table 2), and the results show that the toxicity was highly influence by the particle size. The higher the particle is, the higher the EC20 and EC50 values are.

Table 2 – Effective concentration (g/L) causing 20 and 50% of effect after exposure of the algae *Raphidocelis subcapitata* (Growth inhibition assay) to the three nanoparticles (NP)

Silica Nanoparticles	Size	EC50 (g/L)	EC20 (g/L)
TM40	22 nm	9.32 (6.08 – 13.39)	2.71 (2.15 – 3.44)
HS30	12 nm	9.07 (7.63 – 11.17)	2.09 (1.83 – 2.37)
SM30	7 nm	7.93 (6.50 – 10.01)	1.251.05 – 1.46)

4.4. Discussion

In both studies SiNP has shown size-dependent toxicity, for both organisms the bigger particle was, the less toxic. And the smaller particle was the most toxic, different from our results Casado et al. (2013) uses *R. subcaptata* and *Vibrio fisheri* to evaluated 50 nm and 100 nm SiNP and the results shows no toxicity for those organism. On this paper the silica

used was 22, 12 and 7 nm, significant smaller than the silica in Casado's paper, so we can assume that increasing the size we can produce a non toxic SiNP. Van Hoecke et al. (2008), also used *R. subcaptata* as a bioindicator organism and also confirm a size-dependent toxicity, in his studies he also determined that not only size, but also the surface area are important to determinate the toxicity of nanoparticles. Studies with other algae also shown that silica nano-particles has a size dependent toxicity, Fujiwara et al. (2008) studied SiNP to *Chlorella kessleri* and the 5 nm present a IC50 0.8 ± 0.6%, significantly smaller then the IC50 of our smaller particle (7nm), and the 26 nm particle studied in his paper presented a IC50 31 % bigger than the 22 nm particle studied in this paper. Those studies confirmed that silica nanoparticle present a size dependent toxicity and that the inhibition of growth assay with *R. subcaptata* is a strongly and robust test to assessed the toxicity of nanoparticles on the aquatic environment.

4.5. Conclusions

In conclusion the present study demonstrates that silica nanoparticles toxicity is size-dependent and can cause harm to the marine and freshwater aquatic environment. And if we increase size we may produce a SiNP that is not toxic for the environment.

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