Universidade de Aveiro Departamento de Biologia

Ano 2014

Nuno Mouta Faria da Costa

Short term toxicity of nanomaterials in different development stages of amphibians

Toxicidade de nanomateriais em diferentes estádios do ciclo de vida de anfíbios

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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 Isabel Maria Cunha Antunes Lopes, Investigadora Auxiliar do Centro de Estudos do Ambiente e do Mar e Departamento de Biologia da Universidade de Aveiro.

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palavras-chave Nanotoxicologia, Ecotoxicidade, Nanomateriais, Anfíbios

resumo

A produção de nanomateriais artificiais está em constante crescimento. Os rápidos avanços nesta indústria promovem a introdução de nanomateriais (NMs) no meio ambiente, nomeadamente nos ecossistemas aquáticos. As propriedades específicas que estes compostos apresentam podem promover uma maior toxicidade comparativamente aos seus correspondentes de tamanho não nano. Tamanho, carga, área superficial, índice de agregação, entre outras propriedades, podem ditar o grau de toxicidade dos NMs em ambientes aquáticos, especialmente quando combinados com as constantes mudanças de vários parâmetros ambientais, por exemplo pH e temperatura. Os anfíbios são excelentes bioindicadores para estudar o risco associado à introdução de NMs no meio aquático, uma vez que habitam uma grande variedade de habitats de água doce potencialmente contaminados com descargas industriais. O presente trabalho teve como objetivo estudar a toxicidade de NMs em diferentes estágios de vida de anfíbios, tendo em perspetiva a influência do aumento da temperatura global que atualmente afeta o planeta Terra. A fim de alcançar este objetivo, foram realizados dois estudos que pretenderam:

i) avaliar a influência da temperatura na toxicidade de NMs de ácido poliacrílico hidrofobicamente modificado (HM-PAA) para girinos de *Epidalea calamita* e *Pelophylax perezi*. Para tal, girinos de *E. calamita* e *P. perezi* foram expostos a uma gama de seis concentrações de HM-PAA e a um controlo, a temperaturas de 20ºC e 25ºC. Os resultados mostram toxicidade letal e sub-letal provocada pelo HM-PAA, no entanto, não foi visível um padrão claro de influência da temperatura na toxicidade deste NM;

ii) determinar a influência do tamanho de Si-NMs e da temperatura na sua toxicidade para embriões de *Pelophylax perezi*. Ovos de *P. perezi* foram expostos a uma gama de seis concentrações de três Si-NPs com diferentes tamanhos (SM30-7nm, HS30-12nm, e TM40-22nm) e a um controlo, a temperaturas de 20ºC e 26ºC. Os resultados obtidos mostram toxicidade letal e sub-letal causadas pelos 3 NMs e um aumento da toxicidade com temperaturas mais elevadas. Mais ainda o NM com menor tamanho apresentou maior toxicidade.

keywords Nanotoxicology, Ecotoxicity, Engineered nanomaterials, Amphibians

abstract The production of engineered nanomaterials is rising and constantly growing. The fast advances in this industry are causing the introduction of nanomaterials (NMs) into the environment, namely into aquatic ecosystems. The specific properties that these new compounds exhibit may promote higher toxicity to biota, comparatively to their bulk counterparts. Size, charge, surface area, aggregation index, among others, may dictate the availability and the degree of toxicity of NMs in aquatic environments, especially when assembled with environmental changing conditions such as pH and temperature. Amphibians are excellent bioindicators to study the risk associated with the release of NM into the aquatic environment, since they inhabit a wide variety of freshwater habitats associated with industrial contamination. The present work intended to study the toxicity of NMs to different life stages of amphibians, concerning the increase of global temperature that is currently taking place. In order to achieve this, two specific goals were determined:
i) evaluate the influence of temperature in the toxicity of NMs of hidrophobically modified polyacrylic acid (HM-PAA) to tadpoles of *Epidalea calamita* and *Pelophylax perezi*. For this, tadpoles of *E. calamita* and *P. perezi* were exposed to a range of six concentrations of HM-PAA plus a control, at 20ºC and 25ºC. Results showed lethal and sublethal toxicity of HM-PAA, but a clear pattern of temperature influence in the toxicity of HM-PAA could not be unveiled;
ii) assess the influence of Si-NM size and temperature in the toxicity of this NM to embryos of *Pelophylax perezi*. To attain this goal, embryos of *P. perezi* were exposed to a range of six concentrations of three differently sized Si-NMs (SM30-7nm, HS30-12nm, and TM40-22nm) plus a control, at 20ºC and 26ºC. Results obtained show lethal and sublethal toxicity caused by all the Si-NM and an increased toxicity at higher temperatures. Furthermore, it was observed that the NM presenting the lowest primary size exhibited the highest toxicity.

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Main Introduction

Nanomaterials – General

Nanotechnology has revolutionized the industrial world with the development and production of a large range of nanomaterials (NM). Currently, several recommendations for the definition of nanomaterials exist at an international context (e.g. USEPA, 2007; EC, 2011; Health Canada, 2011). In the present work, the definition recommended by the European Commission (recommendation 2011/696/EU; EC, 2011) will apply: "a natural, incidental or manufactured material containing particles, in an unbound, aggregated or agglomerated state and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %."

These materials, nano scale sized, exhibit a unique set of physical and chemical properties that allows their efficient use and applications in a wide sort of fields. In fact, the ability to manipulate these characteristics has raised a great interest from the nano industries, which increased research and production of NMs at a global level. Nanomaterial's commercial applications may almost be considered unlimited, including the cosmetic industry (e.g. NMs of titanium dioxide and zinc oxide used in sunscreens; NMs of iron oxide in lipsticks, NMs of alumina and protein-based NMs in soaps, shampoos and detergents), environmental field (e.g. as catalysts for the remediation of industrial effluents), automotive (e.g. fullerene nanotube composites in tires), clothing, pharmaceuticals, food industries, among many others (Biswas and Wu, 2005; Rosário, 2012).

Nanomaterials have always existed in the environment and can derive both from natural or anthropogenic sources (Nowack and Bucheli, 2007). Volcanic eruptions, forest fires or even simple erosion are some of the natural processes that originate NMs emission (Rosário, 2012). However, anthropogenic sources such as manufactured NMs, unwanted industrial by-products of combustion, welding or chemical manufacturing are introducing new NMs into the environment (Moore, 2006; Liden, 2011; Farré et al., 2011; Rosário, 2012). Indeed, the mass development and production of engineered NMs has led to its inevitable appearance and availability in different environmental compartments: levels of 0.06 ng/m^3 and 0.48 ng/m^3 aerosol-bound fullerenes were registered in the Mediterranean

atmosphere (Sanchís et al., 2012); Mitrano et al. (2012) reported levels of 200ng/L and 100ng/L of Ag-NM in the influent and effluent, respectively, of a wastewater treatment plant, thus predicting its discharge to the aquatic ecosystems, among other examples in the literature (e.g. Maurer-Jones et al., 2013; a review published by Gottschalk et al., 2013). Though these concentrations are quite low, with the increasing production of NMs it is expected that their concentrations in the environment will also increase.

As with classical chemical compounds, NMs started to be developed, produced, and incorporated into consumer products before an adequate understanding of its behavior, fate and toxicity in the environment, i.e., without carrying out prospective risk assessments on them. But, since the late 2000's an increasing concern on the possible adverse effects that NMs may pose to both environmental and human health arose in the scientific community and, since then, a boom of scientific works addressing these issues occurred (Kahru and Dubourguier, 2009; Ivask et al., 2014). These concerns ground specially in the unique properties of these new compounds, for example their nano size, highest surface:volume ratio, higher reactivity, which may confer them a higher potential to cause adverse effects to biota than their bulk counterparts (Maurer-Jones et al., 2013). Despite of the existing literature, no consistent picture has arisen yet, while some works report that bulk materials are more toxic than the corresponding NMs, other works report the opposite or the inexistence of differential toxicity. For instance, Musante and White (2010) observed that NM of Ag were more toxic that bulk Ag; they reported adverse effects of 100mg/L of NM of Ag to the biomass and transpiration of *Cucurbita pepo* seedlings while the same amount of the bulk Ag powder exerted no effects at a similar concentration. These authors, also reported equivalent phytotoxicity of NM of Cu and the corresponding bulk material at 100 and 500mg/L. However, Stampoulis et al. (2009) observed that bulk Cu powder caused a reduction of 69% in the biomass of *C. pepo* while exposure to NM of Cu resulted in a reduction of biomass of 90% relatively to the control. The former authors, suggest that these different outcomes could be related with the fact that they tested lower concentrations of the NM and that at such low concentrations the particle size dependency to the NM's toxicity was decreased. Besides this factor (NM concentration) many other have been pointed to influence the fate, behaviour and toxicity of NMs. In fact, depending on the characteristics of the media, specific properties of NM, like size, surface charge, reactivity, may change and alter its interactions with natural/non-natural existing compounds and its risk to biota (Elsaesser and Howard, 2012; Cornelis et al., 2014). Musante and White (2010) observed that in the presence of humic acids the ion content of bulk Cu solution decreased, while it provoked an increase in ion content originated on NM-Cu in solutions by factors of 1.4 to

2.9 times, but, while the toxicity of the former solution decreased, the toxicity of the latter one was not significanlty altered, and Fabrega et al. (2009) reported that the stability of suspensions of NMs of Ag increased in the presence of humic acids and that these changes impacted the toxicity of the NM to bacteria when occurred at pH=9. Furthermore, the size of NMs is a determining factor of reactivity, transport, and toxicity (Maurer-Jones et al., 2013). It is commonly accepted that smaller particles cause significantly higher toxicity than larger particles (Napierska et al., 2009). The ionic strength of aquatic media may influence the size of NM and consequently their toxicity, since engineered NMs tend to aggregate more when the ionic strength of media is higher (Remédios et al., 2012).

Described adverse effects caused by NMs in aquatic organisms go from generation of reactive oxygen species (ROS), neurotoxic effects, DNA damage, reproduction impairment, somatic growth inhibition, incidence of malformations to increased mortality rates (Adams et al., 2006; Lopes et al., 2012; Mouchet et al., 2008; Nations et al., 2011a, 2011b; Thit et al., 2013; Van Hoecke et al., 2008; Wong et al., 2010). Salvaterra et al. (2013) reported induced oxidative stress in *P. perezi* tadpoles, when exposed to $TiSiO_4$ NMs. In a study reporting lethal and sublethal toxicity of ZnO NMs, Wong et al. (2010) reported a significant up-regulation of superoxide dismutase (SOD) in five aquatic species: marine diatoms *Skeletonema costatum* and *Thalassiosia pseudonana*, crustaceans *Tigriopus japonicus* and *Elasmopus rapax*, and the medaka fish *Oryzias melastigma*. Biochemical marker analysis represents an essential tool when assessing the exposure and effect of NMs in the aquatic environment (Moore, 2006), since it has been suggested that enzymatic activity is very sensitive, occurring before observed adverse effects at the individual level. As environmental concentrations of NMs are usually low, while not leading to visible effects in aquatic biota at the individual level, can generate neurotoxicity and oxidative stress to the organisms (Maurer-Jones *et* al., 2013).

At this point, it is important to understand the behavior of NMs in natural ecosystems and try to predict the impacts associated to nano-bio interactions in order to assure sustainable use of this technology.

Nanomaterials within the context of Climate Change

The latest report of the International Panel for Climate Changes (IPCC; Fourth Assessment Report) states that “Climate change in IPCC usage refers to a change in the state of the climate that can be identified (e.g. using statistical tests) by changes in the mean and/or the variability of its properties, and that persists for an extended period, typically decades or longer. It refers to any change in climate over time, whether due to natural variability or as a result of human activity…” (Barker, 2007). Currently, global climate change is considered one of the major conservation threats to natural ecosystems. Climate has indirect influence in most of the natural species with temperature, precipitation and extreme natural events causing variations in habitat complexity, food supply and species typical behavior (McCarty, 2001; Pearson and Dawson, 2003). According to the IPCC, the warming from 1850–1900 to 2003–2012 (the most recent decade) was 0.78 [0.72 to 0.85] °C and temperature is projected to increase 1.8–4.0°C by the end of the century. The report also states that 20-30% of the plant and animal species evaluated so far in climate change studies are at risk of extinction if temperatures reach such expected levels (Hartmann et al., 2013). In addition to the direct effects in the biota, another consequence of climate change is the potential to alter the environmental distribution and biological effects of contaminants. Some works already shown that fluctuations in climate can alter physico-chemical and biological dispersal of pollutants between atmosphere, water and soil (Macdonald et al., 2005; Noyes et al., 2009; Schiedek et al., 2007). Temperature and precipitation are the most altered parameters by climate change and tend to have the largest influence in chemical compounds, eventually changing their toxicity in the environment (Blaustein et al., 2003; Schiedek et al., 2007; Noyes et al., 2009). Greater frequency of rain and storm events promote the wet deposition of contaminants in terrestrial and aquatic systems. Storm waters incorporating chemical compounds can spread across ground and surface water by runoffs, causing diluted contaminants to accumulate in benthic sediments. Through higher accumulation rates, toxic concentrations scale up, increasing the potential uptake to benthic biota (Chiovarou and Siewicki, 2008).

Looking at the global increase of temperature, several authors have been trying to understand the effects and risks of temperature rise and its interactions with chemical contaminants to natural biota (Blaustein et al., 2003; Hatch and Blaustein, 2000; Macías et al., 2007; Rumschlag et al., 2014; Sanuy et al., 2008). Some, believe that the combined

effects of natural and chemical stressors can be one of the reasons increasing the declining of some species (Blaustein et al., 2001; Hooper et al., 2013; Iii et al., 2006; Lushchak, 2011; Mann et al., 2009; Noyes et al., 2009; Schiedek et al., 2007). Aquatic ecosystems are amongst the most affected by chemical contamination due to the endless transport routes for contaminants into this environmental recipient. Chemical compounds as NMs, pesticides or metals can travel through the water column, deposit and accumulate in the bottom sediments or submerge become suspended in the water column (Farré et al., 2008; Klaine et al., 2008), increasing the risk of uptake to a wide range of aquatic organisms. Temperature can alter the properties of these toxic compounds, increasing their toxicity in the water (Greco et al., 2011; Maurer-Jones et al., 2013; Osterauer and Köhler, 2008; Peralta-Videa et al., 2011; Schiedek et al., 2007). Studies relating temperature increase with chemical contamination cover the effects on freshwater species such as fish, bivalves and amphibians (Osterauer and Köhler, 2008; Greco et al., 2011; Hooper et al., 2013). Described effects can go from oxidative stress, suppression of immunological responses, neurotoxicity, molecular and cellular interactions to developmental malformations, eventually leading to increased mortality rates (Elsaesser and Howard, 2012; Greco et al., 2011; Lydy et al., 1999; Osterauer and Köhler, 2008).

Climate events will continue to take place, altering global ecosystems and changing the behavior of contaminants. Bearing the above in mind, and revisiting the previous section, it is clear the urgent need to understand the toxicity of NM under more ecologically relevant lab-exposure scenarios for the yield of better reports, allowing better environmental protection.

The use of amphibians in combined toxicological studies

The extended loss of amphibian populations is a worldwide concerning subject. Individual cases of population declines point to several potential causes, habitat destruction being the most noteworthy (Laurance et al. 2002, Eterovick et al. 2005, Cushman 2006; Tsuji-Nishikido and Menin, 2011). Numerous causes, including pathogens, introduced non-native species and global environmental changes are contributing to population declines around the world (Blaustein and Kiesecker, 2002; Blaustein et al., 2003). Because amphibian x ndecline is already a significant problem, it is essential to understand how climate change affects these individuals (Corn, 2005). Temperature is one of the primary factors influencing larval growth and development in amphibians and can become lethal when interacting with

other existing stressors in the natural environment (Boone and Bridges, 1999). Variations in temperature and moisture can trigger early breeding activity (Araújo et al., 2006), alter hibernation periods and foraging activity (Blaustein et al., 2001); increasing temperature can lead to pond desiccation, exposing larvae to UV-B and increasing the risk of fungal and parasitic lethal infections in water-dependent species (Blaustein et al., 1994; Blaustein and Wake, 1995; Hays et al., 1996; Kiesecker et al., 2001; Blaustein et al., 2003).

Amphibians are very susceptible not only to subtle climate changes but also to pollution and can act as bioindicator species for environmental contamination (Gardner, 2001). There is a growing evidence that chemical contamination is, in some way, responsible for amphibian declines (Blaustein et al., 2003). Permeable skins, unprotected eggs, biphasic life stages and inhabiting the boundary of aquatic and terrestrial environments, makes amphibians highly susceptible to all kinds of pollutants (Duellman and Trueb, 1994; Vallan, 2000), especially embryos and newly metamorphosed larvae (Vitt et al., 1990). Adults constitute a prey for both aquatic (e.g. fish, macroinvertebrates) and terrestrial (e.g. birds) biota (Santos, 2011). The biphasic life cycle and unique physiology, suggests that this group has additional opportunities for exposure, via different routes, to environmental contamination than other vertebrate groups (Mann, 2000).

Combining chemical stress with environmental events, introduced species and habitat destruction we can hypothesize why amphibians are becoming endangered: synergistic effects between stressors. Researchers found that there is not a single primary cause for global declines, instead all of these factors are co-operating, threatening amphibian populations (Blaustein and Kiesecker, 2002). Within the frame of emergent pollutants, like NM, it is expected that this group of organisms will have to deal with contaminants other than those traditionally considered. In fact, as tadpoles feed at the surface of sediments and NM tend to aggregate when in aqueous media and settle, it is expected that this life stage of amphibians will constitute an ecological receptors that will be more exposed to NM, and, therefore, will be one of the ecological receptors at higher risk.

Objectives

Accordingly to the above, the present work aimed at studying the influence of increased temperatures in the toxicity of two types of nanomaterials in early-life-stages of amphibians. Two specific objectives were delineated:

(i) Assess the role of temperature in the toxicity of nanomaterials of hydrophobically modified crosslinked polyacrilic acids to tadpoles of *Epidalea calamita* and to *Pelophylax perezi*. This specific objective will be addressed in chapter 1.

(ii) Determine the influence of temperature and size in the toxicity of silica nanomaterials to embryos of *Pelophylax perezi*. This specific objective will be addressed in chapter 2.

This study was carried out in strict accordance with the recommendations present in the Guide for the Care and Use of Laboratory Animals of the European Union – in Portugal represented by the Decreto Lei n°113/2013 de 07 de Agosto. Organisms were euthanized through deep freezing at -80ºC, an extremely fast method, minimizing stress and suffering and also not influencing the measure of the biochemical parameters, thus not compromising the achievement of the work objectives. Approval by a named review board institution or ethics committee was not necessary as the final model for ethical experimentation using amphibians as biological models was not implemented in Portuguese research units at the time of experimentation. This work was conducted under an institutional license for animal experimentation and a personal license to Isabel Lopes, issued by the Direcção Geral de Veterinária (DGV), Portuguese Ministry of Agriculture, Rural Development and Fisheries. All necessary permits for egg collection of *Pelophylax perezi*, a non-endangered species, at the field site, not privately owned or protected in any way, were obtained from ICNB-Instituto de Conservação da Natureza e Biodiversidade, the Portuguese regulatory body concerned with the protection of wildlife and responsible for the authorization of animal collection in the field.

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Chapter One

Influence of temperature on the lethal and sublethal toxicity of hydrophobically modified polyacrylic acid on tadpoles of *Pelophylax perezi* and *Epidalea calamita*

Introduction

Nanomaterial manufacturing industries are emerging and quickly evolving. Advances in these industries are pushing the usage of nanomaterials (NMs) into a variety of areas from pharmaceuticals and cosmetics to environmental remediation (Nations et al., 2011a; Remédios et al., 2012). Given the massive production and commercialization of nano-scaled compounds, the potential for their release in the environment and consequent risks to natural ecosystems are becoming major concerns for environmental toxicologists and regulators (Delay and Frimmel, 2012; Farré et al., 2009). Nanomaterials from solid wastes, wastewater releases or even industrial accidental spillages can migrate to soils, surface and ground waters by wind or rainwater runoffs, contaminating natural water systems and aquatic biota (Klaine et al., 2008). Once in the environment, NM properties may suffer several transformations, such as size and surface area variation due to aggregation/agglomeration, dissolution or dispersion (Guzman et al., 2006; Wiesner et al., 2006). These changes may be induced by both abiotic and biotic environmental factors, like, for example UV-B, pH, temperature, organic matter, which may change NM behavior in the environmental matrices and as well their toxicity to biota, by for example increasing its rate of uptake by the organisms (Levard et al., 2012; Navarro et al., 2008; Nel et al., 2006; Schiedek et al., 2007).

Studies with aquatic organisms have demonstrated that the presence of engineered NMs in a medium can cause adverse effects as reduced fertility, abnormal behavior, malformations and increased mortality rates (Lovern and Klaper, 2006; Templeton et al., 2006; Roberts et al., 2007; Salvaterra et al., 2013). Nations et al. (2011) reported abnormalities in the development after exposing *Xenopus laevis* larvae to four NMs: Fe_2O_3, TiO_2, ZnO and CuO NMs. In addition, studies with *Daphnia magna*, *Hyalella azteca* and two fish species, *Pimephales promelas* and *Oryzias latipes* showed that carbon-based NMs may cause significant sub-lethal effects (Oberdorster et al., 2006). Zhang et al. (2012) revealed that TiO_2 NMs can increase the production of reactive oxygen species (ROS) and induce oxidative stress in *Xenopus laevis* tadpoles, when combined with UV light exposure. Other NMs, such as of Ag and fullerenes, also showed the ability to produce ROS upon UV exposure (Badireddy et al. 2007; Rodriguez-Moya 2007). In a study that evaluated the effects of fullerene nanocrystals (nC60) to *Daphnia magna* under different medium conditions, Tao et al. (2011) showed that water hardness favors nC60 aggregation, leading to higher uptake ratios. Uptake was also related with increased pH and temperature levels.

Daphnid filter frequency increases with higher temperatures, maximizing particle uptake (Heugens et al., 2006).

Although a lot of studies focus on NMs effects to standard freshwater organisms, there is a lack of knowledge regarding the effects they pose to native species. *Xenopus laevis* is commonly used in NMs toxicity assays as an amphibian model species (Haywood et al., 2004; ASTM, 2007; Nations et al., 2011a, 2011b; Thit et al., 2013; Zhang et al., 2012). Still, model species maintained under controlled laboratory conditions could possibly exhibit different sensitivities from those species inbreeding in natural ecosystems. Furthermore, heading ecological relevance, the impact of abiotic factors in the toxicity of NMs should also be addressed. The global increase of temperature is one of the aspects that can change species behavior in the wild (Beebee and Griffiths, 2005; Corn, 2005; Pounds, 2001). Many species were able to adapt and survive through these events, but the accelerated increase of temperature is unprecedented and faster than predicted by climate change reports (Bickford et al., 2010; Wake, 2007). According to the International Panel for Climate Change report of 2013, the globally averaged combined land and ocean surface temperature data shows a warming of 0.85 [0.65 to 1.06]°C, over the period 1880–2012 (Hartmann et al., 2013). Increased environmental temperatures can lead to pond desiccation, reduction of soil moisture, embryo mortality, shifts in mating and metamorphosis periods and affect sex ratios of amphibian offspring (Araújo et al., 2006; Bickford et al., 2010; Blaustein et al., 2003; Blaustein et al., 2001; Corn, 2005).

Accordingly, the aim of this study was to evaluate the influence of increasing temperature on the lethal and sublethal toxicity of a NM of crosslinked hydrophobically modified polyacrylic acid-HM-PAA to tadpoles of two species of amphibians - *Pelophylax perezi* and *Epidalea calamita*.

Material and Methods

Studied NMs

Nanomaterials of hydrophobically modified crosslinked polyacrylic acids (HM-PAA) with short and long aliphatic chains, located in the interior of the NM, were here studied. These NMs, are commonly used in industry due to their unique properties to induce thickening of liquids. For this reason they are mostly applied in inks, paper, pharmaceuticals, and cosmetic products (Antunes et al., 2011). A stock suspension of 30%

of HM-PAA was manufactured and provided by the Chemistry Department of the University of Coimbra (Coimbra, Portugal).

Nanomaterial concentrations, used in the ecotoxicity assays, were obtained by direct dilution of the stock suspension with FETAX medium (chemical composition: NaCl, $NaHCO_3$, KCl, $CaCl_2$, $CaSO_4 \cdot 2H_2O$, $MgSO_4$; ASTM, 1998).

NMs characterization

For all tested concentrations of HM-PAA, the hydrodynamic diameter (as a measure of the NM size), polydispersion index and zeta potential (as a measure of dispersion stability and surface charge) were characterized using the techniques of dynamic light scattering (DLS) and electrophoretical light scattering (ELS) in a Malvern Zetasizer Nano ZS (ASTM 1985; Malvern Instruments 2008)

Test species

Two species of amphibians were selected to carry out this work: the toad *Epidalea calamita* and the frog *Pelophylaz perezi*. Eggs of *E. calamita* were collected at a reference temporary shallow pond, located in the Alto Guadalquivir region, southern Spain (''El Ardal''; 38º 08'N 3º 35'W). Eggs of *P. perezi* were collected at a permanent pond located in Quinta da Boavista, Central Portugal (40º 36'N 8º 41'W). Both sites have no history of chemical contamination. Eggs were collected in Gosner stage 10-11 and transported to the laboratory in plastic containers filled with local water. In the laboratory, eggs were cleaned from debris, transferred to the artificial medium FETAX, and maintained with constant aeration and photoperiod of 14:10h (light:dark), at 23 ± 1°C, until hatching and reaching Gosner stage 25, stage used to perform ecotoxicity assays.

Ecotoxicity assays with tadpoles

The ecotoxicological assays were conducted according to the FETAX standard protocols for tadpole assays (ASTM, 1998).

Tadpoles in Gosner stage 25 were exposed, for 168 h, to six concentrations of the HM-PAA (1.8; 2.8; 4.1; 6.2; 9.3; and 14 mg/L) and to a control (FETAX medium), under laboratory controlled conditions of constant aeration, temperature and photoperiod of 16:8h (light:dark). Five tadpoles were randomly introduced in 500 ml plastic recipients filled with 250 mL of test solution (NM concentration or FETAX). For each NM concentration and control, four replicates were performed. Exposure to the six concentrations of HM-PAA and to the control occurred at the temperatures of 20 ± 1°C and of 25 ± 1°C. During exposure, food was added daily as 20 mg of Tetramin (*ad libitum*), medium was replaced every 48 h.

The following parameters were measured in the renewed and new media: pH (pH 330/SET-2, best nr. 100 788), conductivity (LF 330/SET, best nr. 300 204), dissolved oxygen (OXI 330/SET, best nr. 200 232) and ammonia levels (DR 2000 Spectrophotometer method 8038 for water wastewater and seawater - © Hach Company 1991-1993).

Malformations and mortality of organisms were monitored each 24 h, and whenever an organism died it was removed from the test vessel to avoid media degradation.

At the end of the assay, all tadpoles that were alive were measured and weighted using a magnifying glass (Leica MS5) at a 10x factor and an analytical balance (Kern, ABS), to acquire total body length (TBL) and total weight (TW). Afterwards, the tadpoles were deep frozen in liquid nitrogen and rapidly stored at -80°C for further biochemical analysis.

Biochemical analysis

The following biochemical markers were analyzed to address the occurrence of oxidative stress: catalase (CAT) and glutathione S-transferase (GST).

Each tadpole was homogenized separately using a sonifier (Branson S-250A), in 1.2 ml of potassium phosphate buffer (0.1M; pH 7.4). At least four replicates were made for each treatment. The homogenized samples were centrifuged (Eppendorf, 5810 R) for five minutes and the supernatant collected. The protein content in the supernatant obtained from centrifuged samples was calculated using the Bradford method (Bradford, 1976), adapted to microplate, at 595 nm, using bovine γ-globulin as standard. For determination of CAT, GST and protein content a Labsystem Multiskan EX microplate reader was used as follows:

Catalase

Catalase activity was determined by the method described by Clairbone (1985). It consists in following the decrease in the absorbance at 240 nm, which represents the decomposition of H_2O_2 (substrate) in H_2O and O_2.

Glutathione S-transferase

Glutathione S-transferase activity was quantified based on the method described by Habig and Jakoby (1981) and adapted to the microplate by Frasco and Guilhermino (2002). GST combines the substrate 1-chloro-2.4-dinitrobenzene (CDNB) with glutathione, leading

to the production of a tioether, which can be monitored by the increase in absorbance at 340 nm.

Data analysis

The software SigmaPlot v.11 was used to perform data analysis of CAT, GST and ChE activities. A two-way analysis of variance (ANOVA) followed by the Tukey test was performed to evaluate significant differences between treatments ($p<0.05$). Data normality and homogeneity of variances were tested with the Shapiro-Wilks and Bartlet's tests, respectively. Data values considered outliers were withdrawn from analysis. Outlier points were values higher or lower than the mean plus or minus two times the standard deviation.

Results

Physico-chemical parameters of the test media did not varied significantly throughout the assay. Dissolved oxygen was always above 6.6 mg/L and conductivity between 500 and 700 µS/cm. Ammonia levels did not exceed 0.9 mg/L and pH values ranged from 7.5 in higher treatments to 8.4.

Regarding the physical parameters of NM, it was observed that their size significantly changed with concentration, *id est*, average hydrodynamic diameter increased with increasing concentrations: the lowest value measured was 760 nm at 1.8 mg/L and the highest 2050 nm at 14.0 mg/L ($p<0.001$; Table 1). This, suggests that at higher concentrations, the NMs present in the suspension exhibit a greater tendency to flocculation and consequent sedimentation.

No significant differences were observed among the values of PDI measured for the six concentrations of HMPAA ($p= 0.443$). These values were within the range of 0.219 and 0.337, indicating heterogeneity of particles' size (Table 1).

Though significant differences were registered among the values of zeta potential ($p<0.05$) they were all within the category of good stability (> |30mV|; ASTM, 1985): -39.6 mV at concentration 4.1mg/L and 49.6mV at concentration 14.0mg/L (Table 1).

Table 1 – Physical parameters measured at the six tested HMPAA concentrations. PDI stands for polydispersity index. [a,b,c,d,e,f] - represent homogeneous groups ($p<0.05$) within each measured parameter.

HM-PAA	Z-Average (nm)	PDI	Z-Potential (mV)
1.8	760 ± 15.4[a]	0.221 ± 0.154	-40.9 ± 1.04[a]
2.8	1104 ± 34.0[b]	0.219 ± 0.095	-43.0 ± 3.38[a,b]
4.1	1273 ± 29.7[c]	0.287 ± 0.088	-39.6 ± 0.434[a]
6.2	1571 ± 42.7[d]	0.267 ± 0.117	-43.5 ± 1.10[a,b]
9.3	1805 ± 60.3[e]	0.245 ± 0.110	-43.4 ± 0.920[a,b]
14.0	2050 ± 44.5[f]	0.337 ± 0.091	-49.6 ± 1.20[b]

Responses at the individual level

No mortality was registered in the control groups with FETAX medium. At the end of the assay and for the temperature of 25ºC, mortality rates were lower than 10% for both species. Higher mortality occurred at the temperature of 20ºC for *E. calamita* and *P. perezi*: LC_{10} [3.08 (1.43-4.47)] mg/L and LC_{10} [6.60 (1.70-2.10)] mg /L of HM-PAA, respectively. Though it was not quantified, comparatively to the tadpoles in the control with normal movement, tadpoles exposed to the highest NM concentrations (9.3mg/L and 14.0mg/L, both for *E. calamita* and *P. perezi*) showed a decrease in mobility and unusual swimming behavior. Malformations were not observed at the end of the assay, for any of the exposed tadpoles.

Temperature did not influence TBL or TBW in control tadpoles, since significant differences were not observed in these parameters for tadpoles exposed to the control under 20ºC or 25ºC ($p<0.05$; Figs. 1 to 4).

Regarding TBL, no significant interactions occurred between temperature and the tested concentrations of HM-PAA for *E. calamita* and *P. perezi* ($p\geq0.240$; Fig. 1 and 2).

A significant decrease in TBL was only observed at 20ºC for tadpoles of *E. calamita* exposed to HM-PAA concentrations of 1.8 mg/L and 9.3 mg/L, comparatively to the respective control ($p<0.05$; Fig. 1).

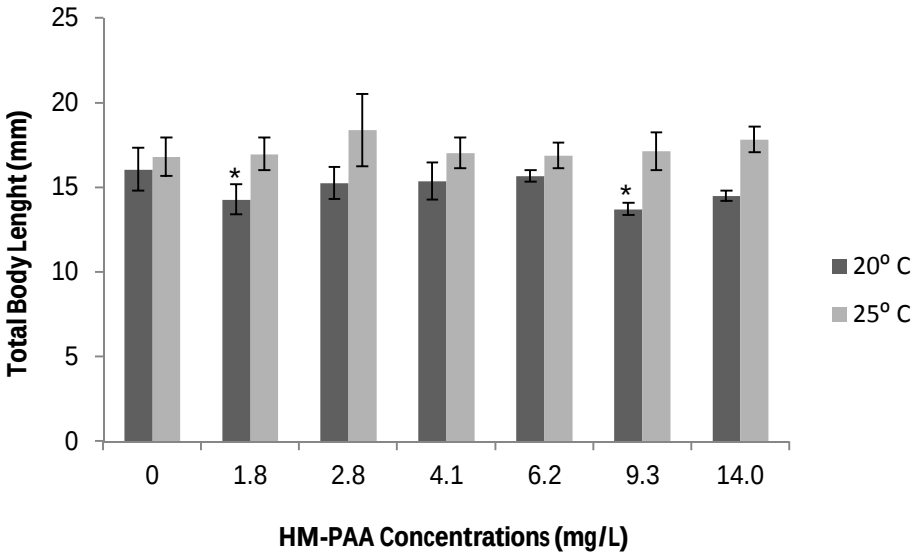

Figure 1 - Average of total body length (mm) for *E. calamita* tadpoles after being exposed for 168h to six concentrations of HM-PAA. * represent significant differences between HM-PAA concentrations and the respective control ($p<0.05$). Error bars represent standard deviation.

The tested concentrations of HM-PAA did not exert significant effects on the TBL of *Pelophylax perezi* at the two temperatures (Fig. 2; $p=0.665$).

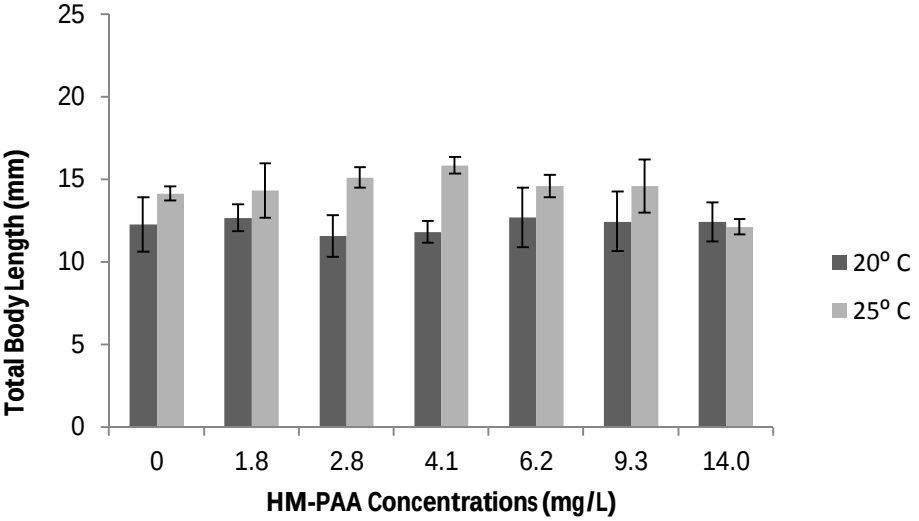

Figure 2 - Average of total body length (mm) for *P. perezi* tadpoles after being exposed for 168h to six concentrations of HM-PAA. Error bars represent standard deviation.

Regarding TBW, a significant interaction between temperature and HM-PAA concentrations was only observed for *E. calamita* ($p=0.019$; Fig. 3). Though no significant differences were observed in TBW of tadpoles exposed under controlled conditions to the

two temperatures, when exposed to HM-PAA the TBW of tadpoles was always higher when under 25ºC, comparatively to when exposed to 20ºC ($p<0.05$; Fig. 3). Furthermore, a significant effect of HM-PAA was only observed at 25ºC for 2.8mg/L of HM-PAA that caused a significant increase in TBW comparatively to the respective control ($p<0.05$; Fig. 3).

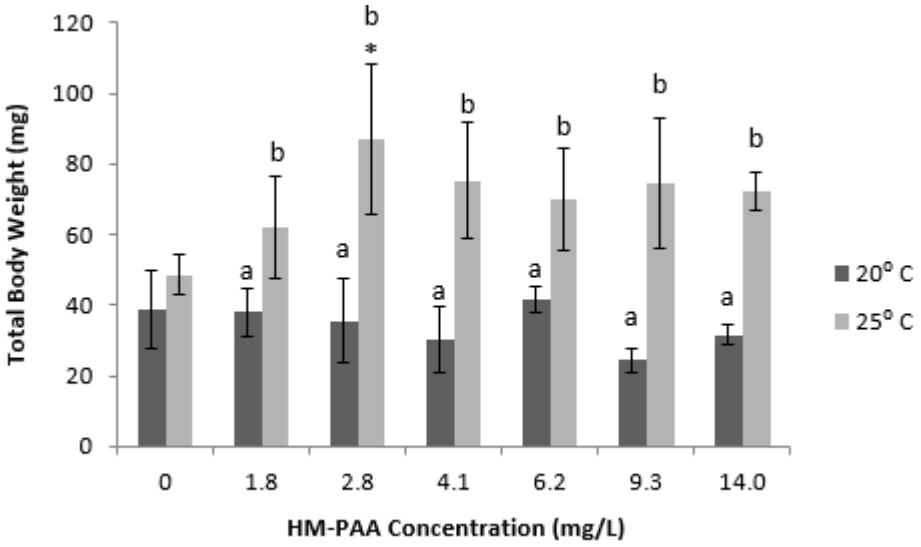

Figure 3 - Average of total body weight (mg) of *E. calamita* tadpoles exposed for 168h to six concentrations of HMPAA. a,b represent significant differences between temperatures and *- significant differences between HM-PAA concentrations and the control ($p<0.05$). Error bars represent standard deviation.

For *P. perezi* no significant differences were observed between TBW of tadpoles exposed under 20ºC or 25ºC ($p<0.05$; Fig. 4). Exposure to HM-PAA only significantly affected TBW, by increasing it comparatively to the respective control, of tadpoles exposed under 25ºC to 4.1mg/L of HM-PAA ($p<0.05$; Fig. 4).

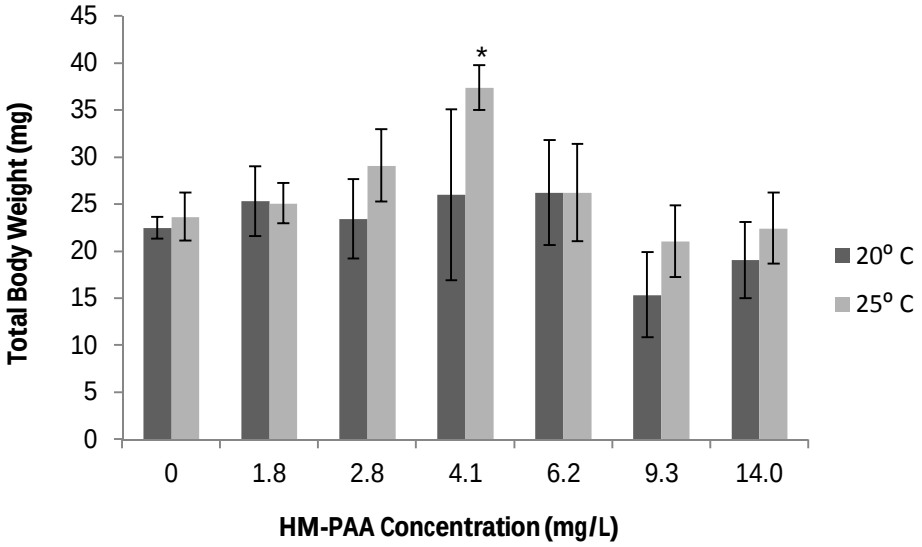

Figure 4 - Average of total body weight (mg) of *P. perez*i tadpoles after being exposed for 168h to six concentrations of HMPAA. * represent significant differences between HM-PAA concentrations and the control ($p<0.05$). Error bars represent standard deviation.

Responses at the biochemical level

Exposure to HM-PAA significantly affected both GST and CAT activity for the two species studied.

For *E. calamita,* there was a statistic significant interaction between temperature and HM-PAA ($p<0.001$). Tadpoles exposed to HM-PAA, at 20ºC, exhibited a significant decrease in CAT activity comparatively to the respective control in nearly all concentrations. However, at 25ºC, the activity of CAT increased at the two lowest concentrations (1.8 and 2.8 mg/L) of HM-PAA comparatively to the respective control (Fig. 5).

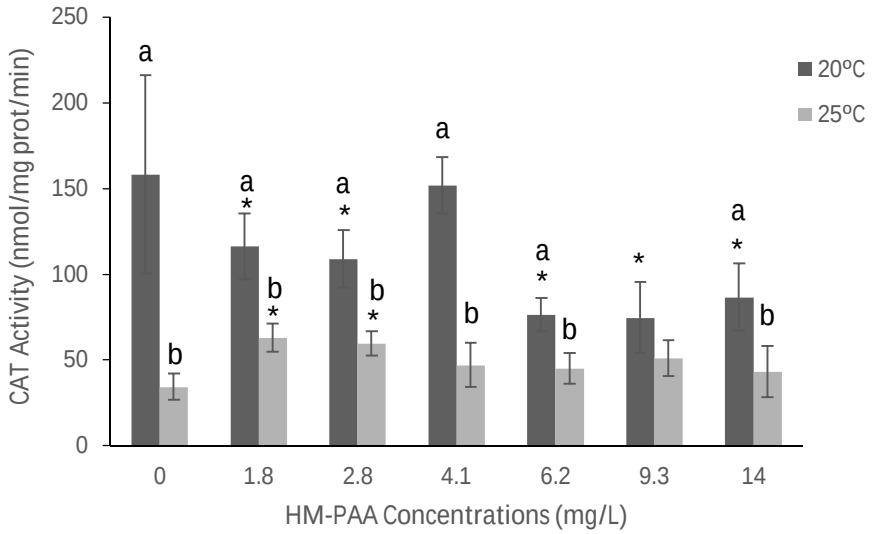

Figure 5 - Catalase activity in *E. calamita* tadpoles. a,b - significant differences between temperatures and * - indicates significant differences between HM-PAA concentrations and the respective control ($p<0.05$). Error bars represent standard deviation.

For *P. perezi* temperature showed no significant interactions with HM-PAA ($p>0.05$). At 25ºC, higher levels of CAT activity were observed when compared with 20ºC results ($p<0.05$). Catalase activity showed a significant increase for the highest concentrations of HM-PAA tested (6.2, 9.3 and 14.0 mg/L, respectively; $p=0.03$) at both 20 and 25ºC comparatively with the respective control (Fig. 6).

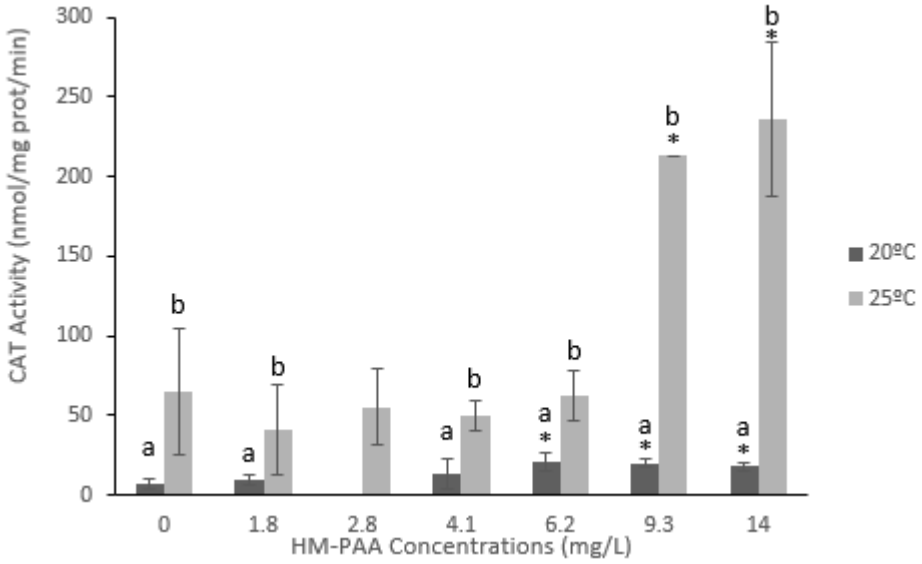

Figure 6 - Catalase activity of *P. perezi* tadpoles exposed to different concentrations of HMPAA. a,b - significant differences between temperatures and * - significant differences between HM-PAA concentrations and the control ($p<0.05$). Error bars represent standard deviation.

Regarding GST activity, it was significantly affected by HM-PAA in *E. calamita* (Fig. 7). At 20ºC for the concentrations of 6.2, 9.3 and 14.0 mg/L, GST activity levels were significantly lower than those in the control ($p\leq0.047$), whereas at 25ºC all HM-PAA concentrations provoked a decrease in the activity of GST ($p<0.05$). For the concentration of 1.8mg/L results show an influence of temperature in HM-PAA toxicity.

The activity of GST for *P. perezi* could not be quantified due to insufficient protein content.

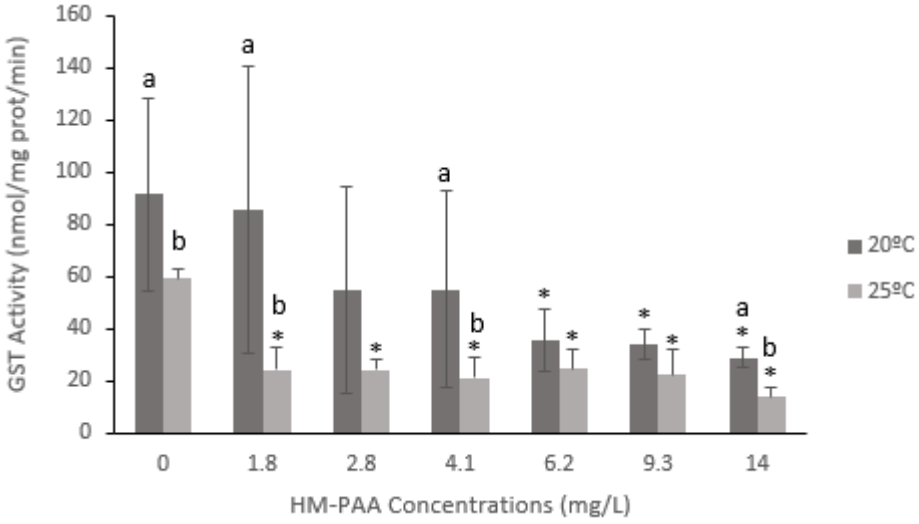

Figure 7 - GST activity in *E. calamita* tadpoles. a,b - significant differences between temperatures and * - significant differences between HM-PAA concentrations and the control ($p<0.05$). Error bars represent standard deviation.

Discussion

Amphibians have shown to be highly sensitive to both industrial contaminants (Barry, 2011; Lopes et al., 2012; Nations et al., 2011a, 2011b; Santos, 2011; Thit et al., 2013) and to climatic fluctuations (Bickford et al., 2010; Blaustein et al., 2011; Hooper et al., 2013).

In this study, we aimed to assess the influence of increasing temperatures on the behavior of NMs of HM-PAA and their toxicity to two species of amphibians. First the characterization of the HM-PAA physical properties was performed, since they are crucial when assessing toxicity in biological systems (Jiang et al., 2008; Oberdörster et al., 2000). Size, morphology, intrinsic reactivity and agglomeration index are among the central parameters that need to be evaluated to assess NM behavior in the suspension (Donaldson et al., 2004; Renwick et al., 2004). These parameters are all related with NM potential risk, since smaller particles are more reactive and disposed to agglomeration, thus representing higher toxicity (Albanese et al., 2012).

The NMs of HM-PAA aggregated when suspended in FETAX medium. This was an expected result since, due to the ionic strength of the media, the double layer at the surface of NMs decreases leading to a reduction in the electrostatic repulsive forces between the particles in suspension and, thus, increasing the probability of aggregation (Suttiponparnit

et al., 2011). Actually, this type of behavior has been reported for a wide panoply of NMs when suspended in aqueous suspensions (e.g. Pereira et al., 2011; Lopes et al. 2012).

Furthermore, it was observed that the hydrodynamic diameter of HM-PAA, dispersed in FETAX, increased with increasing concentrations. This could be due to the fact that at higher concentrations the probability of particles of HM-PAA encountering each other is higher, as their double layer is reduced due to the ionic strength of the media, they would tend to aggregate more. Other authors already reported such differential aggregation associated with NM concentration. For example, Karlson et al. (2003) showed that the HM-PEG polymers (hydrophobically modified v polymer) tend to form flower-like micelles, which aggregate in clusters at higher concentrations. The other physical parameters measured for the NM did not change significantly. PDI always showed heterogeneity in the size range of NMs in suspension and the potential zeta always sowed a good stability of the suspensions.

It was expected that fluctuations in temperature could enhance the toxic effect of HM-PAA to the tadpoles, as it is known that higher temperatures increase the metabolic rates, which could lead to higher uptake and ingestion rates. The obtained result showed higher mortality rates at 20ºC for *E. calamita* and *P. perezi*, when comparing with exposures at 25ºC. These results, suggest that temperature indeed influence HM-PAA toxicity, though not in the pattern that was expected. According to Lushchak (2011), the decrease in environmental temperature may weaken the reactive oxygen species (ROS) elimination system, which increases the probability of occurrence of oxidative stress. On the other hand, increasing temperature can produce physiological stress. At less extreme conditions, increasing temperature is generally related to an increase in metabolic rates, which is particularly evident in ectotherms (Rosa et al., 2012). This, though may lead to an increase uptake of chemicals, also intensifies the activity of the excretory system and increases the role of detoxifying mechanisms that could explain the lower effects observed at the individual level when exposure occurred at 25ºC (Lydy et al., 1999; Mann et al., 2009; Rumschlag et al., 2014). Also, Liu et al. (2011) reported that the increase in temperature may affect NM stability, facilitating aggregation and decreasing the NM availability in a medium.

Temperature alone did not exert a clear influence in TBL for *E. calamita*. Although, when HM-PAA was added, TBL showed a significant decrease for the concentrations of 1.8mg/L and 9.3mg/L, at 20ºC. No differences were observed for TBL in *P. perezi*. In addition to causes related with the intrinsic sensitivity to chemicals of each species, this could be related to the fact that these two species were collected at different regions,

involving a different history of exposure to higher temperatures. *Epidalea calamita* was collected at the interior of southern Spain where mean temperatures are much higher than those registered in coastal regions of Central Portugal, where *P. perezi* was collected. If the population of *E. calamita* is acclimated to higher temperatures, then exposure to lower temperatures, as explained above, could lead to a higher sensitivity to HM-PAA. In addition, Noland and Ultsch (1981) stated that shallow water breeder such as toads, could be better adapted to high temperatures in microenvironments than permanent water forms such as frogs.

For TBW, results showed an increase in tadpole weight for both species at 25ºC. This could be due to a faster development of tadpoles as a response to exposure to chemical contamination, in order to move away from the contaminated water body earlier (Gross et al., 2007). In this study the total body length was measured from the tip of the head to the tail, but the body length (without the tail) of the organisms was not measured. These tadpoles exhibiting higher body weight in the presence of HM-PAA could hold smaller tails but bigger bodies, due to a faster development, thus explaining the higher body weight. In order to achieve better results, body length (nose tip to cloaca) should be considered. Beasley et al. (2012) reported differences in developmental delay patterns in *Danio rerio*, when exposed to silver NMs at different temperatures, but according to Peltzer et al. (2013) and McDaniel et al, (2008), faster metamorphosis of amphibian larvae exposed to contamination may be explained as a consequence of stress. This accelerated development, which led to an increase in TBW, could be explained by the interactions between temperature and HM-PAA toxicity, as observed to *E. calamita* at 25ºC.

Regarding biochemical results, for *E. calamita*, both CAT and GST activity were significantly higher at 20ºC, proving that temperature, individually, might have an influence in the enzymatic activity and could, on its own, cause oxidative stress in amphibian species, as already referred by Lushchak (2011) and Ross et al. (2012), for coral species.

Significant differences in CAT activity were obtained in almost all concentrations of HM-PAA at 20ºC. This could mean that HM-PAA showed higher toxicity at 20ºC, especially with 6.2, 9.3 and 14.0mg/L resulting in oxidative stress and decreasing CAT activity. Catalase activity reduction has already been related to oxidative stress in other studies (Atli et al., 2006; Ferrari et al., 2008; Lushchak, 2011). In a study assessing oxidative stress responses, Falfushinska et al. (2008) observed a depletion of CAT activity in *Rana ridibunda* populations after exposure to pesticides. Ferrari et al. (2008) obtained similar results, when studying the responses of *Rhinella arenarum* embryos to pesticide exposure. At 25ºC it was observed an increase in CAT at the lowest concentrations of HM-PAA (1.8mg/L and

2.8mg/L), suggesting that even at low concentrations, HM-PAA may cause oxidative stress in *E. calamita* tadpoles.

For *P. perezi*, temperature seemed to influence CAT activity. The influence of HM-PAA was significantly notorious at both temperatures. Catalase activity increased at the highest concentrations of the compound, showing oxidative stress both at 20ºC and 25ºC. The higher values measured at 25ºC in 9.3 and 14.0mg/L could be a result of the temperature interaction with HM-PAA, which potentiated the effects. At 20ºC, higher levels of CAT were registered for the highest concentrations. This type of response at high concentrations of contaminants has already been reported for other organisms exposed to manufactured NMs, namely daphnids (Klaper et al. 2009). Also, Santos (2011) studied the combined effects of copper exposure and salinity to *P. perezi*, observing both increase and decrease in CAT activity for embryos and tadpoles, respectively. Since CAT is one of the most important enzymes in cell antioxidant defense system it is expected an increase in its activity with higher stress levels (Dazy et al., 2009). However, CAT reduction may be associated with high oxidative stress scenarios, where the enzyme may be inhibited by its own substrates or by the direct action of the contaminant (Pigeolet et al., 1990).

For GST in *E. calamita*, levels clearly indicate a decrease when compared to the control group at both temperatures. Higher temperatures (25ºC) combined with HM-PAA toxicity seem to inhibit GST activity in all concentrations. A diversity of chemical mixtures are known to inhibit GST activity due to a general impairment of the chemical metabolism, interfering with mechanisms of GST induction (Lajmanovich et al., 2011). These results agree with those obtained by Klaper et al. (2009), where increasing concentrations of manufactured NMs caused oxidative stress, inhibiting GST activity in *Daphnia spp*. Santos et al. (2011) also reported that copper exposure to *P. perezi* induced a significant decrease in GST activity. This could be a faster response to high HM-PAA concentrations, where antioxidant response is inhibited.

We hypothesized that for the highest concentrations of HM-PAA, higher effects would be detected due to the formation of larger aggregates (as confirmed by the hydrodynamic diameter) that could precipitate and be more available for tadpole ingestion.

In most amphibian species the Gosner stage 25 is the longest larvae stage during the aquatic development phases, where the central nervous system is almost completely developed (Gosner, 1960). Therefore, Gosner 25 tadpoles can be more sensitive to aquatic contamination and neurotoxic stress can be more damaging. Although ChE activity was not quantified, tadpoles showed erratic movements comparatively to the control when exposed to higher concentrations of HM-PAA, possibly corroborating neurotoxic effects.

Altogether, *Pelophilax perezi* results suggest a much superior resistance to HM-PAA. As a species that frequently occupies wetlands connected to agricultural fields or even close to industrial discharge waterways, these individuals are constantly in contact with various pollutants. Various studies have confirmed that populations inhabiting at or close to contaminated areas can develop genetic tolerance to the contaminant (Bridges et al., 2001; Lopes et al., 2008; Jansen et al., 2011; Cothran et al., 2013), therefore supporting our hypothesis that *P. perezi* showed more tolerance to the HM-PAA compound.

Conclusions

The results obtained in this study support amphibian sensitivity to aquatic pollutants in early development stages. This study showed that the two species of amphibians exhibit different sensitivities to the studied NM, indicating that results related with NM toxicity cannot be generalized among this group of organisms. Furthermore, it is suggested that sublethal effects should be seriously taken in consideration when conducting risk assessments, since, in the absence of mortality effects, some contaminants may still severely affect organisms causing oxidative stress or even neurotoxic effects that will weaken the immune system, increasing individual vulnerability. Also, the HM-PAA characterization data highlighted the complexity when assessing the ecotoxicity of NMs. The results showed that these particles could interact with medium components and modify their characteristics, which could influence their effects in organisms. This has to be taken in account because the unpredicted reaction of NMs could be responsible for unwanted environmental impacts. Future studies should take in consideration interactions between chemicals and abiotic (e.g. temperature or pH) factors that limit natural populations. Risk assessment should comprise these interactions realistically, considering, for example, worst case scenarios as cautionary principles.

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Chapter Two

Does temperature and size influence the toxicity of silica (SiO_2) nanomaterials to *Pelophylax perezi* larvae?

Introduction

Nanotechnology has been producing an extensive range of compounds in order to improve performance and achieve widespread functionalities. With a unique set of properties that include size, surface area, surface charge, shape, solubility and aggregation index, engineered nanomaterials (NMs) carry countless benefits to a wide range of industrial grounds (Jiang et al., 2008; Oberdorster et al. 2005a,b; Powers et al. 2006). However, those same properties may confer NMs a higher toxicity comparatively to its corresponding bulk materials. Thus, the toxicity and availability of NMs in the environment became a concerning subject to the scientific community. Anthropogenic sources such as contaminated groundwater and soil remediation, unwanted industrial by-products of combustion, welding or chemical manufacturing are pushing NMs into natural ecosystems (Klaine et al., 2008; Lopes et al., 2012; Rosário, 2012).

After being released into the environment, NMs may go through alterations due to their interactions with abiotic and biotic environmental factors (Hooper et al., 2013). Environmental factors such as temperature, pH or salinity are known to influence the toxicity of several chemicals, including NMs (Andrew R. Blaustein et al., 2003; Lydy et al., 1999; Macías et al., 2007; Noyes et al., 2009; Relyea, 2006; Remédios et al., 2012; Schiedek et al., 2007). Among these factors, with constant fluctuations, temperature is an element that became important to integrate in ecotoxicological studies since it alters the biological effects of chemicals. Higher temperatures tend to enhance the effect of toxic chemicals, increasing availability and toxicity within natural ecosystems (Hooper et al., 2013; Noyes et al., 2009; Schiedek et al., 2007).

Aquatic systems are amongst the most affected habitats by industrial contamination with intentional wastewater discharges representing massive toxicological risk to aquatic biota. Particles can aggregate, surface area and size may suffer variations, which may alter their toxicity and behavior, and, thus, offering potential routes of uptake by aquatic organisms (Klaine et al., 2008; Moore, 2006; Nel et al., 2006). Recent literature indicates that these compounds can induce adverse effects as DNA damage, oxidative stress and inflammatory responses (Guzman et al., 2006; Klaine et al., 2008; Moore, 2006; Nel et al., 2006; Sharma, 2009; Singh et al., 2009; Wiesner et al., 2006) Card and Magnuson 2010) and that NMs cellular uptake can be size dependent (Albanese et al., 2012; Chithrani et al., 2006). Smaller NMs pass more easily through biological barriers (Salvaterra et al., 2013) and as particle size decreases, surface area increases providing higher toxicity (Napierska et al., 2009; Van Hoecke et al., 2008).

Studies combining the toxic effects of NMs within global warming perspectives are limited, but are essential when trying to understand the interactions between chemical and environmental stressors to biota. Therefore this study focused in the evaluation of the influence of temperature and nanomaterial size of LUDOX Si-based NMs – SM30 (7 nm), HS30 (12 nm) and TM40 (22 nm) – on their lethal and sublethal toxicity to early life stages of *Pelophylax perezi*.

Material and Methods

Nanomaterial characterization

Silica based nanomaterial (Si-NM), electrostatically stabilized with negative charge, with sodium as counter ion and with three different sizes were purchased to Grace & Co-Conn: SM30 (size: 7 nm and surface area: 320-400 m^2/g), HS30 (size: 12 nm and surface area: 220m^2/g) and TM40 (size: 22 nm and surface area: 140m^2/g).

Test concentrations were obtained by directly diluting these stock suspensions with FETAX medium (ASTM, 1998). The two highest tested concentration of each Si-NM where characterized through dynamic light scattering (DLS), to measure the hydrodynamic diameter of particles in suspension (NM size) and polydispersity index (PDI), and by using electrophoretic light scattering (ELS) to measure the zeta potential (measure of stability and surface charge). Measurements were made in a Malvern Instrument Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK) in backward scattering at 173º C. The zeta potential and polydispersity index provided information about the stability and agglomeration of NP in the medium used for all tests.

Test species

The amphibian species *Pelophylax perezi* was selected to carry out the present work. Egg clutches of this species were sampled at a permanent pond located in Quinta da Boavista, Aveiro, Central Portugal (40º 36'N 8º 41'W). Eggs were collected in Gosner stage 10-11 and transported to the laboratory in plastic containers filled with local water. In the laboratory, eggs were cleaned from debris attached to the jelly coat, transferred to the artificial medium FETAX (ASTM, 1998), and immediately used to perform ecotoxicity assays.

Embryo toxicity assays

This assay was conducted according to the FETAX standard protocols for embryo assays (ASTM 1998, 2000). Eggs, at Gosner stage 10-11, were exposed to the following concentrations of each NM and to a control (FETAX medium) for a period of 96h: 0.9, 1.4, 2.1, 3.1, 4.7, 7.0 for SM30 (7 nm); 0.8, 1.2, 1.8, 2.7, 4.0, 6.0 for HS30 (12 nm); and 2.6, 4.0, 5.9, 8.9, 13.3, 20.0 for TM40 (22 nm). Ten eggs were introduced, randomly, in a 60mm Ø Petri dish filled with 10 mL of test solution (NM concentration or FETAX medium). For each concentration and control four replicates were carried out. Exposures occurred at 20 ± 1°C and at 26 ± 1°C under a photoperiod of 16:8h (light:dark). Test medium was replaced after 48h of exposure, and the following physical-chemical parameters were measured in the old and new media: pH (pH 330/SET-2, best nr. 100 788), conductivity (LF 330/SET, best nr. 300 204), dissolved oxygen (OXI 330/SET, best nr. 200 232) and ammonia (DR 2000 Spectrophotometer method 8038 for water wastewater and seawater - © Hach Company 1991-1993).

Dead individuals were removed daily, keeping track of mortality rates. At the end of the assay, all living tadpoles were frozen in liquid nitrogen and rapidly stored at -80°C for further biochemical analysis.

Biochemical markers analysis

The biomarkers measured in this assay were: glutathione S-transferase (GST) and cholinesterase (ChE) to address the occurrence of oxidative stress and neurotoxicity, respectively.

Each tadpole was homogenized separately using a sonifier (Branson S-250A), in 1.2 ml of potassium phosphate buffer (0.1M; pH 7.4). At least four replicates were made for each treatment. The homogenized samples were centrifuged (Eppendorf, 5810 R) for five minutes and the supernatant collected for the procedures referred below. The protein content in the supernatant obtained from centrifuged samples was calculated in quadruplicate using the Bradford method (Bradford, 1976), adapted to microplate, at 595 nm, using bovine γ- globulin as standard.

Cholinesterases

The method described by Ellman (1961) and afterwards adapted to the microplate by Guilhermino et al. (1996) was here used to determine the activity of cholinesterases. Acetylthiocholine (substrate) is degraded by ChE into acetate and tiocholine. The latter

interacts with 5,5-dithiobis-2-nitrobenzoic acid (DTNB), originating a yellow liquid that can be monitored by the increase in absorbance at 412nm.

Glutathione S-transferase

Glutathione S-transferase activity (GST, EC 2.5.1.18) was quantified based on the method described by Habig and Jakoby (1981) and adapted to microplate by Frasco and Guilhermino (2002). GST combines the substrate 1-chloro-2.4-dinitrobenzene (CDNB) with glutathione, leading to the production of a tioether, which can be monitored by the increase in absorbance at 340nm.

Data analysis

Student t test was used to compare size, PDI, and Z-potential between the two highest concentrations of each NM. The 96-h concentrations inducing 10, 20, and 50% of mortality (LC_{10}, LC_{20}, and LC_{50}, respectively) and their 95% confidence limits (CL) were calculated through probit analysis, using the software PriProbit (Sakuma, 1998). The software SigmaPlot v.11 was used to perform data analysis of GST and ChE activities. A two-way (ANOVA) analysis of variance ($p<0.05$) followed by the Tukey test was performed to evaluate significant differences between treatments. Assumptions of ANOVA, i.e. data normality and homogeneity of variances, were tested with the Shapiro-Wilks and Bartlet's tests, respectively. Data values considered outliers were withdrawn from analysis. Outlier points were values higher or lower than the mean plus or minus two times the standard deviation.

Results

Nanoparticle charaterization

The hydrodynamic size of the LUDOX NMs increased, when suspended in FETAX medium, comparatively to the primary size.

When comparing size between the two highest concentrations, it was observed that NMs exhibited smaller sizes at the highest concentrations ($p<0.05$; Table 2). Though significant differences occurred between the Z-potential of the two highest concentrations of each NM, the values were always above |30| mV, indicating a high stability of

suspensions. Polydispersion values were always above 0.192, indicating heterogeneity of sizes of the NMs in suspension (Table 2).

Table 2 – Physical parameters (average ± standard deviation) measured for the two highest concentrations tested for each nanomaterial of silica.

	Concentration	Z-Average (d.nm)	PDI	Z-Potential (mV)
SM30 (7nm)	4.7	17.0 ± 0.645	0.343 ± 0.013	-42.2 ± 1.13
	7.0	15.4 ± 0.345	0.344 ± 0.006	-35.9 ± 2.18
HS30 (12nm)	4.0	19.5 ± 0.430	0.259 ± 0.007	-23.2 ± 1.29
	6.0	17.2 ± 0.316	0.216 ± 0.003	-33.0 ± 1.98
TM40 (22nm)	13.3	29.5 ± 0.504	0.192 ± 0.007	-34.7 ± 1.88
	20.0	27.3 ± 0.540	0.218 ± 0.006	-42.0 ± 3.49

Effects at the individual level

The lowest observed effect concentration (LOEC) for mortality, after 96h of exposure to SM30 and HS30 were 0.9 mg/L and 0.8 mg/L, respectively. As for TM40, values of 2.6 mg/L and 40.mg/L corresponded to both NOEC (no observed effect concentration) and LOEC.

For the three NM tested, mortality rates were always higher when exposure occurred at 26ºC (Table 3). The values of LC_{10}, LC_{20}, LC_{50} computed at 20ºC were at least 2.8-fold higher than the same parameters computed at 26ºC for SM30 and HS30. For TM40, such difference never exceeded 1.5-fold.

Among the three NMs, SM30 and TM40 exhibited the lowest and highest values of LC_{10}, LC_{20}, LC_{50}, respectively, both at 20ºC and 26ºC.

Table 3 - Concentrations provoking 10, 20, and 50% of mortality (LC_{10}, LC_{20}, LC_{50}, respectively), and corresponding 95% confidence limits, in early life stages of *Pelophylax perezi* exposed to three nanomaterials for a period of 96h. *- Values outside the range of concentrations that were tested.

NANOPARTICLE	TEMPERATURE (ºC)	LC_{10}	LC_{20}	LC_{50}
SM30	20ºC	2.27 (1.70-2.72)	3.06 (2.51-3.54)	5.41 (4.65-6.68)
	26ºC	0.79 (0.58-0.97)	1.04 (0.82-1.23)	1.93 (1.36-2.05)
HS30	20ºC	4.46 (2.82-11.9)	7.43 (5.00-220)	19.7 (9.09-1047)*
	26ºC	1.55 (1.09-1.91)	2.16 (1.71-2.54)	4.13 (3.46-5.26)
TM40	20ºC	8.95 (6.47-3850)	13.0 (-)	25.8 (-)*
	26ºC	7.99 (4.99-10.0)	10.3 (7.48-12.4)	17.4 (14.4-20.8)

At the end of the assay, differences in development stage between temperatures were observed. Larvae exposed to 25ºC reached Gosner stage 24/25 (100% of alive organisms) at 96h, while larvae exposed to 20ºC developed to Gosner stage 20 (100% of alive organisms).

In the control groups no malformations were registered (Table 4; Fig. 9). Higher temperature significantly increased the occurrence of malformations. The highest percentage of malformations was obtained at 4.7 mg/L of SM30 (50%) and at 20.0 mg/L of TM40 (35%), at 26º C. In these cases, malformations were characterized as abnormal body curvature and abdominal edema (Fig. 9). No malformations were observed for the treatments at 20ºC.

Table 4 – Percentage of malformations in *Pelophylax perezi* tadpoles after 96h exposure to SM30, HS30 and TM40 at 26ºC.

Treatment	Concentration	Percentage (%)
SM30	0.9	0.0
	1.4	0.0.
	2.1	0.0
	3.1	**25**
	4.7	**50**
	7.0	0.0
HS30	**0.8**	**12.5**
	1.2	0.0
	1.8	0.0
	2.7	**17.5**
	4.0	10
	6.0	0.0
TM40	2.6	0.0
	4.0	0.0
	5.9	0.0
	8.9	**17.5**
	13.3	**30**
	20.0	**35**

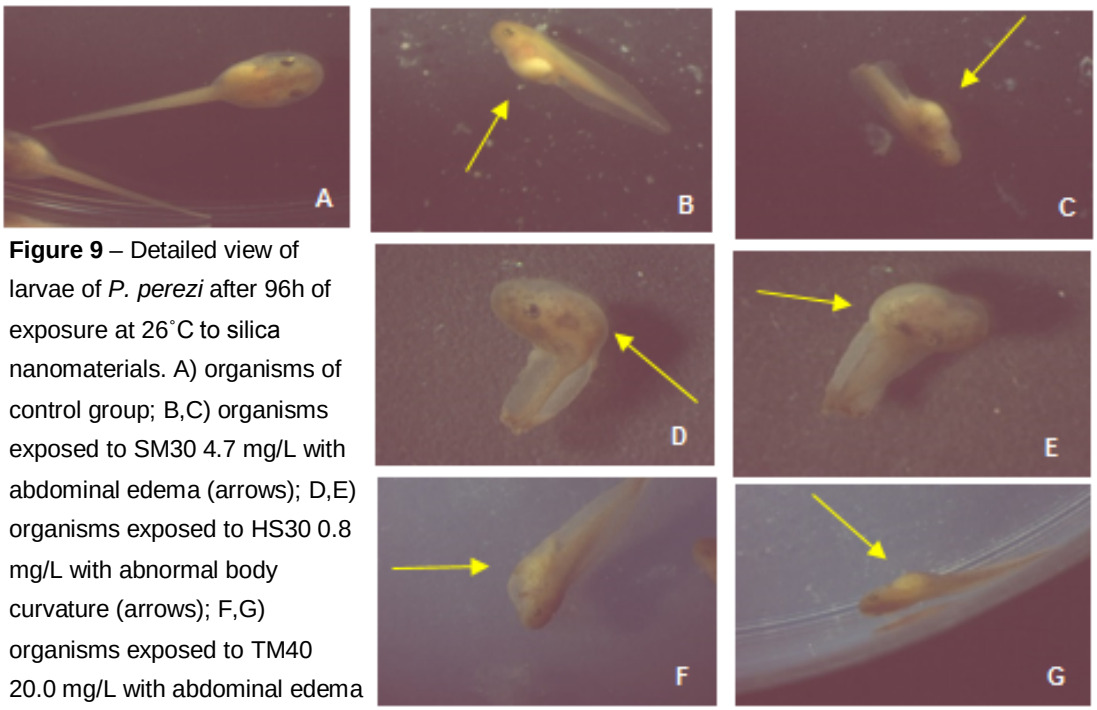

Figure 9 – Detailed view of larvae of *P. perezi* after 96h of exposure at 26˚C to silica nanomaterials. A) organisms of control group; B,C) organisms exposed to SM30 4.7 mg/L with abdominal edema (arrows); D,E) organisms exposed to HS30 0.8 mg/L with abnormal body curvature (arrows); F,G) organisms exposed to TM40 20.0 mg/L with abdominal edema (arrows).

Effects at the biochemical level

When exposed to SM30 concentrations of 1.4 and 3.1 mg/L, the activity of GST was higher at the lowest temperature (20ºC) (p=0.026; Fig. 10).

When exposed to SM30 at 20ºC, GST activity showed a significant increase at the concentrations of 1.4 mg/L and 3.1 mg/L comparatively to the respective control (p<0.05; Fig. 10). At 26ºC, such significant increase in the activity of GST was only observed at 0.9 mg/L, which was the lowest observed effect concentration (LOEC).

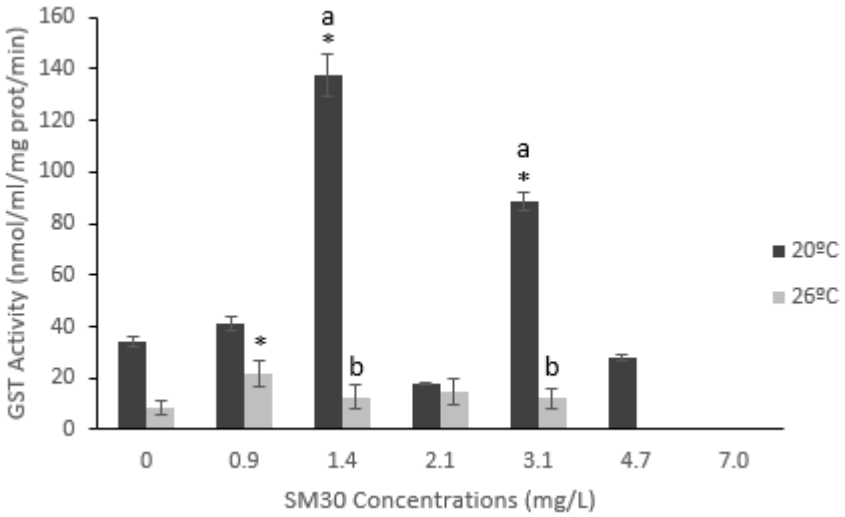

Figure 10 - GST activity in *P. perezi* embryos exposed for 96h to SM30. a,b - significant differences between temperatures; * - significant differences between SM30 concentrations and the respective control (p<0.05). Error bars represent standard deviation.

Exposure to HS30, at 26ºC, did not significantly change the activity of the enzyme relatively to the control. But, at 20ºC, the activity of GST was significantly higher than in the control at the concentration of 4.0 mg/L (p<0.001; Fig. 11). Additionally, the activity of GST at the two highest concentrations (4.0 and 6.0 mg/L) was higher for tadpoles exposed under a temperature of 20ºC (Fig. 11).

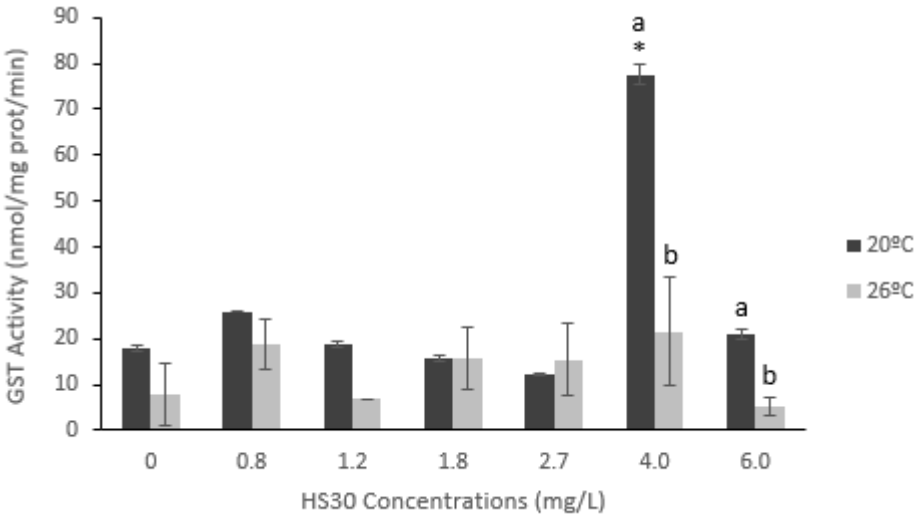

Figure 11 - GST activity in *P. perezi* embryos exposed for 96h to HS30. a,b - significant differences between temperatures; * - significant differences between HS30 concentrations and the control ($p<0.05$). Error bars represent standard

Concerning the exposure to TM40, a significant interaction between temperature and TM40 concentrations was observed ($p<0.001$; Fig. 12). At the concentration of 5.9 mg/L GST activity was significantly higher when exposure occurred 26ºC ($p=0.003$: Fig. 12), while at concentration 20 mg/L GST activity was significantly higher when exposure occurred at 20ºC ($p<0.001$; Fig. 12). Furthermore, it can clearly be observed an increase in GST activity at 26ºC, for concentrations of 4.0; 5.9; 8.9 and 13.3 mg/L, though a decrease at the concentration of 20 mg/L occurred, comparatively to the respective control (Fig. 12). The lowest concentration with effect was 4.0 mg/L.

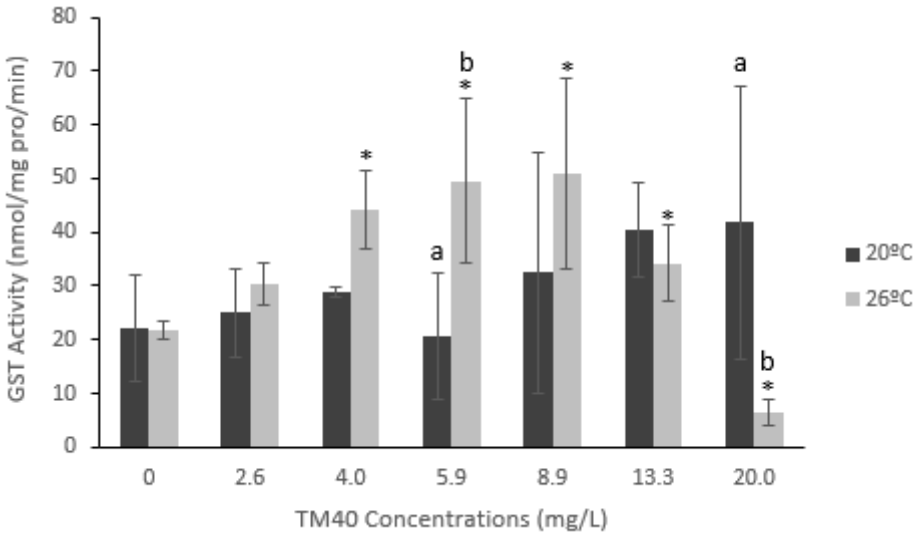

Figure 12 - GST activity in *P. perezi* embryos with TM40. a,b - significant differences between temperatures, * - significant differences between TM40 concentrations and the control ($p<0.05$). Error bars represent standard deviation.

Regarding ChE activity, a decrease, although not significant, was observed at 26ºC with increasing concentrations of SM30. Only at 0.9 mg/L, under exposure at 26ºC, was visible a significant increase in ChE activity when compared with control values (Fig. 13). For the concentrations of 4.7 and 7.0mg/L at 26º, no ChE analysis was made due to insufficient biological sample. At 20ºC no differences in ChE activity were observed.

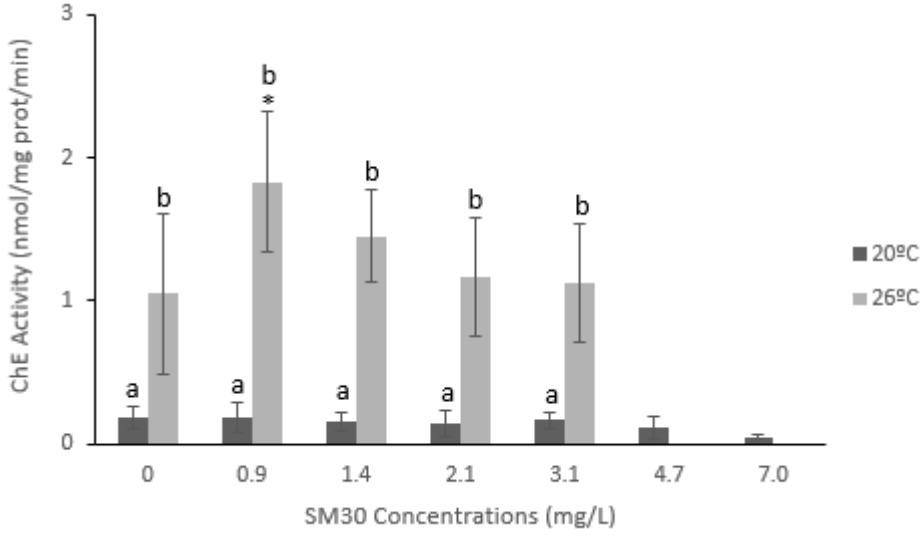

Figure 13 - ChE activity in *P. perezi* embryos exposed for 96h to SM30. a,b - significant differences between temperatures, * - significant differences between SM30 concentrations and the control ($p<0.05$). Error bars represent standard deviation.

Concerning HS30, there was a significant interaction between temperature and HS30 concentrations ($p<0.001$). Cholinesterases activity in mostly all concentrations, showed an increase with exposure at 26ºC comparatively to exposure under 20ºC.

Cholinesterases activity at 26ºC significantly decreased, when compared with control values, at 2.7, 4.0 and 6.0 mg/L ($p=0.026$; Fig. 14). At 20ºC all the concentrations showed significantly lower values than those of the control group ($p= 0.031$), with 0.8mg/L representing the lowest concentration with effect.

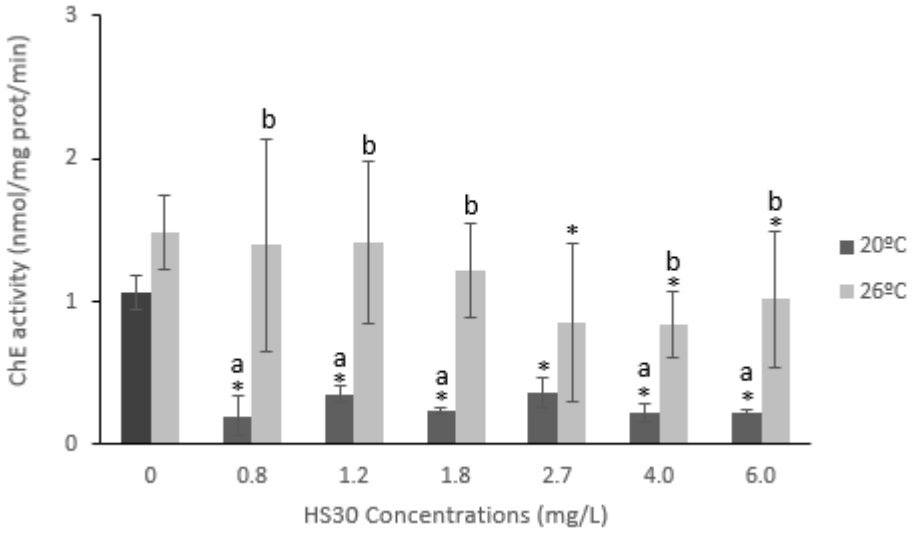

Figure 14 - ChE activity in *P. perezi* embryos after being exposed for 96h to HS30. a,b - significant differences between temperatures, * - significant differences between HS30 concentrations and the control ($p<0.05$). Error bars represent standard deviation.

Finally, for TM40, significant differences were registered at 26ºC for 5.9, 8.9, 13.3 and 20.0 mg/L relatively to 20ºC treatments. It was observed a significant increase in ChE activity from 5.9 to 20.0 mg/L, comparatively to the respective control ($p<0.05$; Fig. 15). In this case, the lowest concentration with effect as 8.9mg/L, one of the highest concentrations tested for TM40.

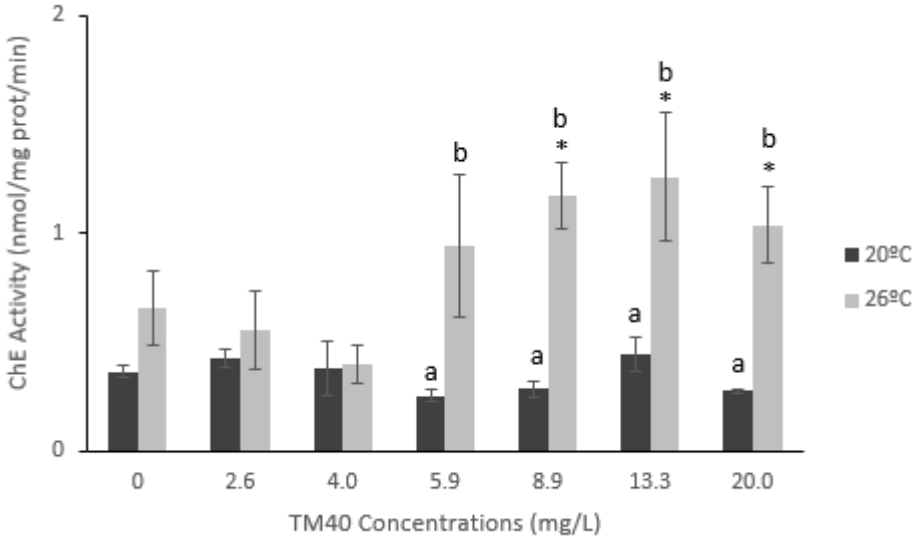

Figure 15 - ChE activity in *P. perezi* embryos after being exposed for 96h to TM40. a,b - significant differences between temperatures, * - significant differences between TM40 concentrations and the control ($p<0.05$). Error bars represent standard deviation.

Discussion

A huge increase of NMs industry, and multiple applications has been noticed (Donaldson et al., 2006; Lewinski et al., 2008; Medina et al., 2007), but the potential for adverse effects, due to prolonged exposure in the environment has not yet been established (Beer et al., 2012; Nagai et al., 2001; Remédios et al., 2012).

In this study it was intended to determine if the toxicity of the LUDOX NMs is size-dependent and if temperature can influence their toxicity.

Regarding the physical parameters that where measured at the two highest concentrations tested for each NM, it was observed an increase in the size comparatively to the primary size. This is in agreement with the data that has been reported in the scientific literature for the occurrence of rapid aggregation of NMs when suspended in aqueous media. This behavior has been reported for a vast range of NMs, including for silica-based NMs (Lopes et al., 2012; Pereira et al., 2011). For example, Van Hoecke et al. (2011) reported the formation of Si-based NM aggregates when this NM was suspended in OECD algal test media and Napierska et al. (2009) also observed the formation of aggregates when NMs of SM30 where suspended in aqueous media. The former researchers also observed high values of potential zeta (above |23|mV), indicating a good stability of the

silica-based NM suspensions, as reported in the present work. Proportionally, NM SM30 aggregated more, since, on average, the size of particles in suspension was more than twice the value of primary size, while for the other NMs the size of aggregates, on average, did not exceeded 1.6-fold the primary size. This result could be explained by the fact that smaller NMs exhibit higher surface areas which increases its reactivity, promoting the aggregation of more particles (Zhang et al., 2014).

Comparing the toxicity of the three NMs, it was observed that the NM exhibiting the lowest primary size, SM30, exerted significant effects at lower concentrations, to the embryos of *P. perezi*, for almost all monitored endpoints, an exception was only observed for ChE at 20ºC. This highest toxicity could be related with the presence of particles with smaller sizes that could more easily cross the biological barriers and enter in the individual and cells provoking adverse effects. In a study assessing LUDOX SM30 cytotoxicity, Napierska et al. (2009) suggested that the modifications in SM30 cytotoxicity could be derived to its aggregation state or to a simple modification of size. Furthermore, she stated that surface area played an important role in cytotoxicity and that finer particles were related with higher cytotoxic effects.

When looking at mortality rates, SM30 (7nm) was the most toxic, causing 100% mortality with the highest concentration of 7.0mg/L, followed by HS30 (12nm) and TM40 (22nm), respectively. LC_{10} and LC_{20} values confirm higher toxicity of SM30 [0.79 (0.58-0.97); 1.04 (0.82-1.23), respectively] and HS30 [1.55 (1.09-1.91); 2.16 (1.71-2.54), respectively].

All the three Si-NMs caused malformations on tadpoles. Malformation percentage increased with higher concentrations for SM30 treatment with 4.7mg/L (50%), followed by TM40 with 13.3mg/L (30%) and 20.0mg/L (35%), although with HS30, malformations could be observed with the lowest concentrations (0.8, 2.7 and 4.0mg/L). According to the results obtained by Salvaterra et al. (2013) to *P. perezi* tadpoles, smaller nanoparticles could pass more easily through biological barriers, possibly making them more toxic than larger ones. In a study with the same Si-NMs, Silva et al. (2012) suggested that smaller nanoparticles were able to penetrate the tested organisms, causing DNA damage, which could be reflected in organism abnormalities and mortality, agreeing with our results.

Temperature interaction with the LUDOX Si-NMs could be observed in the effects at the individual level. Since all malformations occurred at 26ºC, we can assume that this temperature is high enough to increase the toxicity of the LUDOX NMs to *P. perezi*. Also, when observing LC_{50} values, we can clearly see lower values at 26ºC for all three NMs, suggesting that the toxicity of Si-NMs increases with higher temperatures. At the moment

no bibliography regarding the effects of temperature on NM toxicity to natural organisms were found, but some studies have reported an increase of pesticide toxicity with higher temperatures to different aquatic organisms, including amphibians (Boone and Bridges, 1999; Lydy et al., 1999; Osterauer and Köhler, 2008). As NMs properties are influenced by minimum changes in abiotic parameters, it is suggested that temperature increase could alter these specific properties, increasing NM toxicity.

Biochemical analysis is essential when assessing the toxicity of contaminants in aquatic organisms (Rosenbaum et al., 2012). However, the results obtained in the present work did not shown a consistent pattern of response to the different levels of the three NMs. For the SM30 NMs, our results show that temperature did not influenced GST activity. A significant increase of GST activity can be observed with 1.4 mg/L and 3.1 mg/L at normal temperature (20ºC). The low GST activity at 26ºC was not expected, nevertheless, for the concentration of 0.9 mg/L GST activity significantly increased, showing that lower concentrations of SM30 can be more toxic than higher concentrations.

However, according to Klaine et al. (2008) the increase in temperature can possibly increase resistance of organisms to contaminants. For HS30, temperature variation caused changes in GST levels, with differences in concentrations of 4.0 mg/L and 6.0 mg/L. The toxicity of the HS30 NMs was only observed with 4.0 mg/L at normal temperature (20ºC).

At normal temperatures, higher levels of oxidative stress were induced to the tadpoles. In this case, we can hypothesize that the accelerated development of the tadpoles caused by higher temperatures could be also responsible for increasing antioxidant defense mechanisms, thus increasing tadpole resistance to HS30 at 26ºC.

Regarding TM40 results, for this particular NM, GST activity levels were incompatible with those obtained for SM30 and HS30. In this case, the increase in temperature seems to be related with TM40 toxicity. As TM40 concentrations increase, GST activity also increases, reaching its peak with 8.9 mg/L of TM40 at 26ºC. With 13.3 mg/L, GST starts decreasing, reaching the lowest values with 20.0 mg/L. This could be related to high levels of oxidative stress (Santos, 2011) and the size of the NMs could be directly related to its toxicity. Van Hoecke et al. (2008) studied the toxicity of LUDOX TM40 to the green algae *P. subcapitata*. It was observed that the amount of reactive silica was always higher for smaller particles, but that particles were unable to exert toxic effects to this species. Also, TM40 NMs showed little aggregation, appearing as individual particles in the suspension. It is possible that TM40 particles present in the suspension could be more toxic at 26ºC, thus causing neurotoxic effects or that temperature could cause TM40 to aggregate, becoming more available to tadpole ingestion in the bottom.

Concerning ChE activity, HS30 (12nm) resulted in the most toxic NM, inducing neurotoxic effects in all concentrations tested. Once again, temperature caused AChE activity variations, supporting our theory that fluctuations in environmental factors can act as stressors to aquatic organisms (Lushchak, 2011). On the other hand, TM40 induced an increase in ChE activity in the highest concentrations. We can assume that this increase in ChE levels is possibly due to the enhanced effect of higher temperatures on the toxicity of the NMs.

Comparing LOEC values for GST and ChE, the lowest concentration with effect for SM30 was 0.9 mg/L, confirming that SiO_2 NMs can cause oxidative stress and neurotoxicity at low concentrations. Also for HS30, LOEC values comprehended between 4.0 and 0.8 mg/L, showing that this could be a NP that causes serious sublethal effects with low doses. Regarding TM40, LOEC values for GST and ChE at 26ºC were 4.0 and 8.9 mg/L, respectively, agreeing that for aquatic organisms such as *P. perezi* embryos, TM40 can induce severe toxic effects with increasing temperatures.

Conclusions

The results obtained in the present work revealed a size-dependent toxicity of the LUDOX NMs: SM30 NMs were more toxic followed by HS30 and TM40. In general Si-based NMs exhibiting smaller primary sizes exert higher toxic effects to early-life stages of *P. perezi*, which may be related to the fact that the smaller particles present in the suspension can more easily entre the organism and cross biological barriers. Furthermore, temperature also seemed to influence the toxicity of the three tested NMs. For the three NMs, higher mortality and malformation percentages were registered at 26ºC. The patterns of response of GST and ChE were not consistent across NMs concentrations and temperatures, being difficult to establish a cause effect relationship. Nevertheless, according with the obtained results it is suggested that Si-based NM with higher primary sizes should be preferred to be used consumer products, as they constitute lower risk for aquatic biota. Furthermore, when assessing the toxicity of these NMs to derive maximum safe concentrations, temperature should be taken in account, especially within the frame of global climate changes that predicts temperature increases in a near future, and under such scenarios it is expected higher toxic effects of these NMs.

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General Conclusion

The wide use of NMs in consumer products is already leading to its appearance in the environment (Gottschalk et al., 2013 and references therein). However, though in the last decade several works have been carried out to understand the behavior of these compounds in the environment and identify their risks to biota (e.g. Wong et al., 2010; García-Gómez et al., 2014; Zhang et al., 2012), although several uncertainties still persist. Namely, most of the works were performed under laboratory-controlled conditions not taking into consideration abiotic factors that under real scenarios may modify several characteristics of NMs, which in turn may change their behavior and toxicity in the different environmental compartments. The present work was aimed to contribute to fill some knowledge gaps on this research field. Therefore, the influence of increasing temperatures in some physical properties of NMs and in their toxicity to aquatic life stages of amphibians was addressed. This abiotic parameter was selected following the predictions published in the reported of the International Panel of Climate Change (2013), which reiterate a tendency of global temperature rise in the planet Earth in a near future. One of the scenarios described in this report forecast an increase of 1.8 – 4.0°C by the end of the 21st century (Hartmann et al., 2013). Considering also that some works already reported that temperature may control NM aggregation, reactivity and toxicity (e.g. Nowack and Bucheli, 2007; Sharma, 2009), it is clear that the understanding of how temperature increase may influence the fate, behavior and toxicity of NMs is of most relevance.

It was expected that an increase in the temperature would promote a higher toxicity of the studied NMs to the early-life stages of the two amphibian species either due to potential alteration in the toxicokinetics of the compounds or to the impacts on physiological mechanisms of the organisms (Cairns et al, 1975). Actually such pattern of influence has been reported in several published works involving a wide range of chemicals; Lydy et al. (1999) reported an increase in the toxicity of M-parathion, chlorpyrifos and pentachlorobenzene to *Chironomus tentans*; Boone and Bridges (1999) observed that at higher temperatures the insecticide carbaryl provoked lower survival in tadpoles of the frog *Rana clamitans*; Heugens et al. (2003) showed that an increase in temperature caused an increase in mortality rate and the uptake rate of cadmium in *Daphnia magna*, among many other works. Regarding specifically NMs, Hristozov et al. (2009) found that NMs of quantum dots (CdSe/CdS) exerted higher cytotoxicity to cancer cells at higher temperatures.

However, in the present work this pattern on temperature influence in the toxicity of NMs was not consistently observed. In general, in the case of HM-PAA NMs, the opposite was observed, *id est*, higher adverse effects caused by exposure to HM-PAA were observed at 20ºC comparatively to 25ºC. But, some exceptions were observed, though a higher toxicity at lower temperatures (20ºC) for responses measured at the individual level (mortality and total body length) were observed; for total body weight significant effects were only observed at the highest temperature (25ºC). When looking at the biochemical responses a clear relationship between cause and effects could also not be established. Though the pattern of activity of catalase seemed to reveal higher effects at 20ºC, for GST the opposite appeared to occur. For tadpoles of *E. calamita* exposed at 25ºC, a significant increase in catalase activity only occurred at the two lowest concentrations of HM-PAA, while for tadpoles exposed at 20ºC it occurred for almost all HM-PAA concentrations (an exception was at 4.1 mg/L). For *P. perezi*, higher effects were also observed at 20ºC, since a significant increase in catalase activity was registered at concentrations equal or above 6.2 mg/L of HM-PAA; whereas at 25ºC such significant increase was only observed at concentrations equal or above 9.3 mg/L. However, for GST it was observed that tadpoles of *E. calamita* exposed under 20ºC exhibited a significant reduction in the activity of this enzyme at concentrations ≥ 6.2 mg/L; while for tadpoles exposed under 25ºC such significant reduction was observed for concentrations ≥ 1.8 mg/L.

For the Si-based NMs the responses monitored at the individual levels (mortality and malformations) clearly showed a higher toxicity of the three NMs at the highest tested temperature (26ºC). Interestingly, it is worth of notice that for the NM exhibiting the highest primary size the differences between adverse effects caused at 20ºC and 26ºC were smaller than for the other two NMs, thus suggesting that NMs size plays an important role in their toxicity. Actually, this was corroborated by the fact that SM30 (the NMs with the lowest primary size) was the most toxic NM to *P. perezi* followed by HS30 and TM40. Furthermore, this result is in agreement with the data obtained by other works that compared the size-dependency of the toxicity of NMs. As one example, Sawai et al. (1996) reported that the antibacterial activity of NMs of MgO, CaO, and ZnO reduced with increasing particle size.

The biochemical responses of *P. perezi* to exposure to Si-based NMs under different temperatures did not fully mirror the responses at the individual level and varied with the size of NMs. For SM30, the activity of GST increased at 20ºC (1.4 and 3.1 mg/L) and 26ºC (0.9 mg/L) while the activity of ChE only significantly increased at 26ºC (0.9 mg/L). Exposure to HS30 only significantly increased the activity of GST at 20ºC (4.0 mg/L). But, the activity of ChE was significantly affected at 20ºC and 26ºC. In the former case a significant reduction

in the activity occurred at concentrations ≥ 0.8 mg/L, while in the latter a significant decrease in the activity of ChE was only noticed at concentrations ≥ 2.7 mg/L.

Finally, TM40 caused significant effects in the activity of GST only at 26ºC. An increase was registered at concentrations 4.0 to 13.3 mg/L and a decrease at concentration 20.0 mg/L. As well, the activity of ChE was only significantly changed when exposure occurred at 26ºC; an increase was registered at concentrations ≥ 8.9 mg/L.

Accordingly with the above, it was not possible to identify a clear association between responses at individual and biochemical level. Actually, in some cases no effects were observed at the biochemical level, though significant effects were observed at the individual level. This fact, highlights that care should be taken when using biochemical markers as early warnings of adverse effects posed by NMs.

The differences in the influence of temperature in the toxicity of the two studied NMs, namely at the individual level, could be related with their chemical composition. In the case of Si-NMs it could be expected that with increasing temperatures the solubility of Si ions would occur which could cause a higher toxicity of the NMs. Actually, an increase in solubility of inorganic NMs has already been reported for other types of NMs, namely CuO and ZnO. In the case of HM-PAA the solubility of its components is not expected to occur in suspension, thus this effect of temperature would not be influencing its toxicity.

According to the obtained data, it is suggested that risk assessment of NMs should be case specific and for some NMs temperature corrections should be performed when estimating maximum safety levels in the environment.

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