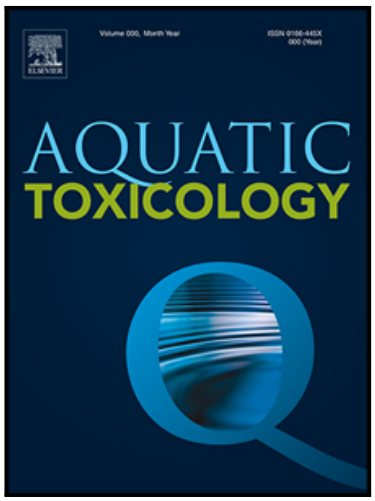

Toxicity of boron and vanadium nanoparticles on Danio rerio embryos – phenotypical, biochemical, and behavioral alterations

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Toxicity of boron and vanadium nanoparticles on *Danio rerio* embryos – phenotypical, biochemical, and behavioral alterations

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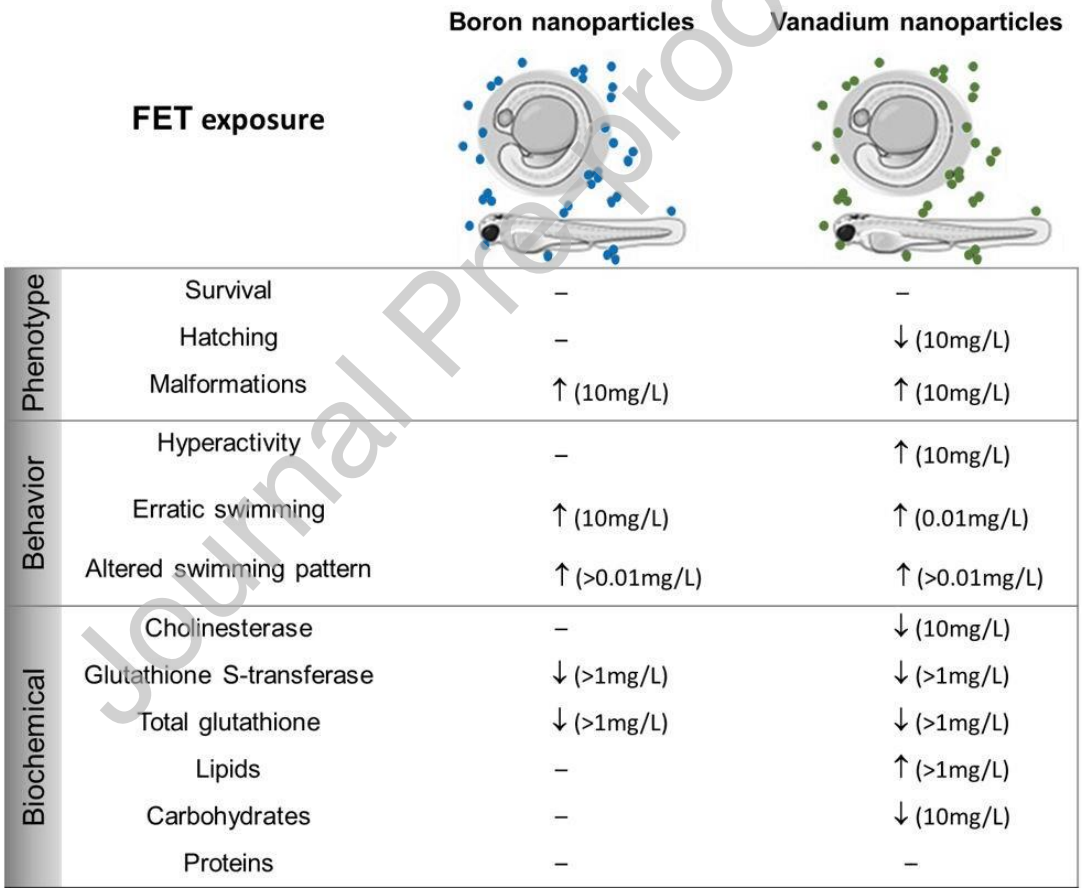
Graphical abstract



Highlights

1. The tested nanoparticles caused dissimilar toxicity mechanisms to zebrafish embryos.

2. Vanadium nanoparticles induced metabolic, neurotransmission, and behavior impairments.

3. Boron nanoparticles affected swimming pattern and induced erratic swimming in larvae.

4. Both nanoparticles induced malformations, such as edemas and spinal malformation.

5. Vanadium and boron nanoparticles may have a negative impact on the aquatic ecosystem.

Abstract

Engineered nanoparticles (NPs) are emerging contaminants of concern and it is important to understand their environmental behavior and ecological risks to exposed organisms. Despite their ubiquitous presence in the environment, there is little information about the hazards of certain NPs, such as boron (BNPs) and vanadium (VNPs). The aim of the present research was to investigate the effects of commercial BNPs and VNPs (80 to 100 nm) to zebrafish embryos, at different levels of biological organization. A range of nominal concentrations for both NPs (0, 0.01, 0.1, 1, and 10 mg/L) was tested. Due to the presence of triton X-100 in the NPs' stock dispersions, an additional control group was included (0.001% triton X-100). Survival, hatching, and malformations of embryos were assessed for 96 hours (h) exposure. Locomotor behavior was evaluated at 120 h. Furthermore, embryos were exposed to 0, 1, and 10 mg/L of NPs to evaluate a set of biomarker responses after 96 h: cholinesterase (ChE) and glutathione S-transferase (GST) activities, total glutathione (TG) and energy budgets levels. VNPs induced malformations (10 mg/L), hyperactivity (10 mg/L), erratic swimming (0.01 mg/L), altered swimming pattern (>0.01 mg/L), delayed hatching (10 mg/L) and altered biochemical

responses involved in antioxidant defense (GST and TG at >1 mg/L), neurotransmission (ChE at 10 mg/L) and energy metabolism (lipids at >1 mg/L and carbohydrates at 10 mg/L). BNPs caused malformations (10 mg/L), affected swimming pattern (>0.01 mg/L), induced erratic swimming (10 mg/L) and decreased TG content and GST activity (>1 mg/L). At the same concentrations, VNPs affected a greater number of endpoints than BNPs, demonstrating a greater toxicity to zebrafish embryos. The present study shows that BNPs and VNPs may affect aquatic organisms, albeit at relatively great non-environmentally relevant concentrations, reinforcing the importance of the risk assessment of different NPs.

Keywords

nanotoxicity; engineered nanomaterials; multi-endpoint approach; zebrafish; risk evaluation; alternative testing

1. Introduction

The nanotechnology revolution has led to an array of applications for nanoparticles (NPs), resulting in their continuous release into the environment (Bakshi, 2020). Therefore, the development of such novel materials should always be coupled with ecotoxicity studies to assess the risk of NPs to the environment and human health (Almeida et al., 2019; Haque and Ward, 2018).

Due to their unique physicochemical properties, metal and metalloid-based NPs, such as vanadium (VNPs) and boron (BNPs), are among the most commonly used (Aksakal and Ciltas, 2019). BNPs are a potential fuel source for liquid fuel engines (Ojha and Karmakar, 2018) and have been applied in

medical research (Strigul et al., 2009). VNPs can be applied in catalysis, electrochromic and optical switching devices, electrochemical capacitors, and windows for solar cells (Aliyu et al., 2017). NPs may be discharged, released, and consequently, accumulated in aquatic ecosystems during synthesis, manufacturing, or use of NP-containing products. Despite the growing interest in their use and applications, little is known about the toxicity of VNPs and BNPs to aquatic organisms. Concerning BNPs, a previous study with *Daphnia magna* showed 100% of mortality for concentrations above 80 mg/L after 24 hours (h) exposure (Strigul et al., 2009). Ecotoxicological studies assessing the effects of VNPs to aquatic organisms were not found.

Elemental boron is present at concentrations ≤0.5 mg/L in surface freshwaters (Çöl and Çöl, 2003) and is considered an essential micronutrient in plants as well as nutritionally important for animals. In fact, the lack of boron in water medium has been shown to adversely affect the embryonic development of some fish species, including *Danio rerio* (Öz et al., 2020). At concentrations below 20 mg/L, boron reduced the adverse effects of oxidative stress in fish via strengthening tissue antioxidant defenses, i. e. by increasing the activities of antioxidant enzymes (Alak et al., 2021, 2020). However, at high concentrations, boron was shown to be toxic and adverse effects have been reported (such as DNA damage, histopathological changes, oxidative stress, growth rate, and feed intake impairments) to different fish species, in particular to *D. rerio* (Alak et al., 2021, 2020; Gülsoy et al., 2015), *Onchorhyncus mykiss* (Alak et al., 2019; Öz et al., 2020), and *Cirrhinus mrigala* (Adhikari and Mohanty, 2012).

Elemental vanadium has been detected at concentrations between 0.010 and 68 µg/L in surface waters (Vasseghian et al., 2021). Vanadium was shown

to induce mortality and malformations in *D. rerio* embryos, namely delayed growth and pericardial edemas (Kim and Lee, 2021). Like other toxic metals, vanadium has the ability to induce the production of reactive oxygen species (ROS), resulting in antioxidant enzyme inhibition and lipid peroxidation (Aureliano et al., 2002). This effect was reported for several fish species including *O. mykiss* (Gillio Meina et al., 2020), *Halobatrachus didactylus* (Aureliano et al., 2002; Soares et al., 2008), and *Clarias batrachus* (Bishayee and Chatterjee, 1994). Other effects on fishes were also described including inhibited growth, damage to the neurological system and to specific organs (kidney, liver, and heart), as well as changes in hematological, reproductive, and respiratory systems (Borges et al., 2003; Fazio et al., 2019).

The zebrafish (*Danio rerio*) has been widely used as a ecotoxicological model organism because of several favorable characteristics (Bai and Tang, 2020). These include a short life-cycle, easy culture in the laboratory, transparency of embryos, rapid development ex utero, high fecundity, availability of extensive genomic information, and genetic homology to higher vertebrates, including humans (Bai and Tang, 2020; Pereira et al., 2019). Besides, the Fish Embryo Acute Toxicity (FET) test is considered as an alternative to animal experimentation according to European Union legislation for the protection of animals used for scientific purposes (Embry et al., 2010; Scholz et al., 2008).

Zebrafish were previously employed to assess the toxicity of several NPs, including metal-based NPs containing gold (Au), silver (Ag), copper (Cu), titanium dioxide (TiO_2), aluminum trioxide (Al_2O_3), copper oxide (CuO), nickel oxide (NiO), and zinc oxide (ZnO) (Bai and Tang, 2020; Haque and Ward,

2018). NPs may accumulate in zebrafish embryos, mainly in the region of the chorion and can then be transported to different organs, mostly the gastrointestinal system, heart, brain, yolk, and liver (Pereira et al., 2019). The toxicity of NPs depends on, among other factors, the physicochemical properties of the NPs, including size, shape, charge, and surface coating (Mendoza and Brown, 2019). In general, NPs have been show to induce ROS formation, oxidative stress, damage to lipids, proteins, and DNA in exposed zebrafish (Mendoza and Brown, 2019; Pereira et al., 2019).

The present study aimed to identify and elucidate the effects of BNPs and VNPs on zebrafish embryos across different organizational levels. For a more complete assessment of the effects of these two NPs, a multilevel approach was applied contributing to an in-depth hazard assessment in the ecotoxicological model organism zebrafish. Individual level endpoints that were considered included survival, behavior (based on larvae locomotion), and morphology (based on embryo development). Furthermore, several sub-organismal endpoints were also included such as biomarkers of antioxidant response (enzymatic and non-enzymatic), neurotransmission, and energy budgets.

2. Material and Methods

2.1. Test organism

Zebrafish (*D. rerio*) eggs were obtained from a culture maintained at the Department of Biology, University of Aveiro, Portugal. Zebrafish adults were kept in a recirculating system with reverse osmosis and activated carbon filtered tap water, complemented with instant ocean synthetic salt automatically

adjusted for pH and conductivity. The fish were maintained at 26 ± 1ºC, under a 12:12 h light/ dark photoperiod cycle, with conductivity at 750 ± 50 µS/cm, pH at 7.5 ± 0.5, salinity at 0.35, and dissolved oxygen at 95% saturation. Adult fish were fed daily with commercially artificial diet Gemma Micro 500 (Skretting®, Spain).

Reproduction groups were placed in an aquarium with marbles at the bottom, in the afternoon of the day before eggs were collected. Two hours after the start of illumination, in the next morning, the eggs were collected and cleaned from residues. Zebrafish eggs (4 hours post-fertilization) with normal development were selected (using a Stereoscopic Zoom Microscope – SMZ 1500, Nikon) for the test. Unfertilized, irregular, or injured eggs were discarded.

2.2. Test nanomaterials and characterization

Commercial BNPs and VNPs (concentration: 20 g/L; purity: 99.9%; CAS number: 7440-42-8 and 7440-62-2, respectively) were acquired from Nanoshel UK Limited. According to the supplier, NPs have a mean diameter of 80 to 100 nm and they were dispersed in ultrapure water containing the surfactant triton X-100 (at 2%). Although it was not provided by the manufacturer, we estimated the mass of each element within a single particle and obtained the values 4.1×10^{-15} g and 5.2×10^{-16} g for boron and vanadium, respectively. From the commercial NPs dispersions, we performed a 100 times dilution in ultrapure water to obtain a working dispersion at 0.2 g/L of NPs (0.02% of triton X-100). Afterwards, the working dispersions of BNPs and VNPs were used to obtain the final test concentrations. Both NPs (in both working dispersions and experimental media) were characterized by hydrodynamic size assessed by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern) and by zeta

potential (ZP) evaluated by electrophoretic light scattering (Zetasizer Nano ZS, Malvern), at 0 and 96 h (test start and end, respectively).

2.3. Quantification of boron and vanadium

The determination of boron (B) and vanadium (V) in the experimental media was performed by inductively coupled plasma mass spectrometry (ICP-MS) using an iCAP™ Q ICP-MS equipment at 0 and 96 h (Thermo Fisher Scientific). Experimental media was diluted with 2% (v/v) nitric acid (HNO_3) containing scandium (Sc) for internal standardization. The elemental isotopes 11B and 51V were monitored for analytical determination; 45Sc was used as internal standard.

2.4. Fish Embryo Acute Toxicity (FET) Test

The FET test was based on the OECD guideline number 236 (OECD, 2013). Zebrafish embryos were exposed to 0, 0.01, 0.1, 1, and 10 mg/L of BNPs and VNPs, and kept at 26 ± 1°C. Each experimental condition consisted of one microplate in which eggs were placed individually into wells containing 2 mL test solution (n=20).The NPs concentration range was based on 10-fold increases, starting with 0.01 mg/L, considered a predicted environmental concentration (Giese et al., 2018). Due to the presence of triton X-100 on the NPs dispersions, an additional experimental condition was included, a surfactant control group (triton X-100 at 0.001%). The concentration 0.001% of triton X-100 used at surfactant control group corresponds to the maximum concentration of triton X-100 used in the assays (for the highest NPs tested concentration: 10 mg/L). The used triton X-100 concentration was ten times lower than the maximum value recommended by the guidelines for most of the

commonly used solvents (0.01%) (OECD, 2017, 2013). Although the available data about the toxicity of surfactants are sparse, previous studies already evaluated the toxicity of triton X-100 for different organisms (including zebrafish embryos and adults). They reported adverse effects of triton X-100 at much higher concentrations (Dayeh et al., 2004; Jang et al., 2007; Kovriznych and Urbancikova, 2001; Oleszczuk et al., 2015) than the ones used in the current study. Oleszczuk et al. (2015) tested the toxicity of various surfactants to *D. magna* and triton X-100 was characterized by the lowest toxicity. The FET test lasted 96 h and embryos were daily observed using a Stereoscopic Zoom Microscope (SMZ 1500, Nikon) to assess mortality, hatching, and the appearance of malformations.

2.5. Locomotor behavior assay

The FET test exposure was extended until 120 h, and larval locomotor behavior was analyzed using the Zebrabox tracking system (Viewpoint Life Sciences, Lyon, France) over a period of 12 minutes (min). Dead larvae or larvae exhibiting malformations were not included in the behavior assay. Ten replicates (individual larvae) per experimental condition were used. The temperature was maintained at 26 ± 1°C and larvae movement was stimulated by alternating light and dark periods. The test consisted of a cycle with two periods: 6 min light and 6 min dark. Total time swimming and total distance swimming by larvae in each period was recorded. In order to measure effects on swimming pattern, total distance, and total time swimming were also recorded in two distinct areas: internal and external zones of the well. Larvae path angle was calculated through the vector of fish swimming direction and the turn path performed by larvae. The angles of movements were grouped in 4

classes, as described by Zhang et al. (2017): class 1 includes large amplitude angles (90-180º), classes 2 and 3 include medium amplitude angles (30-90º and 10-30º, respectively), and class 4 includes small amplitude angles (0-10º) – Figure 1. Three types of movements were considered: low velocity for movements below 8 mm/s; medium velocity for movements between 8 and 40 mm/s, and high velocity for movements above 40 mm/s.

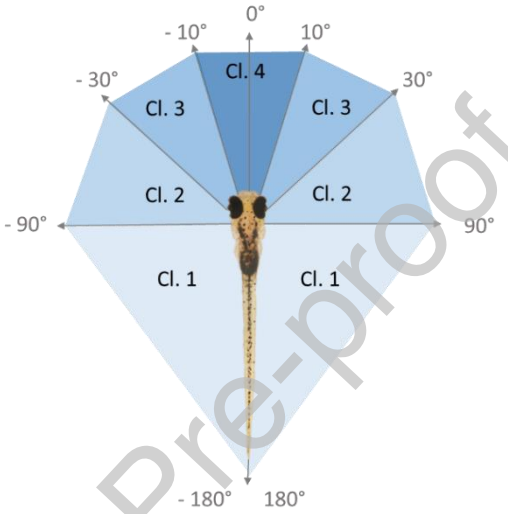


Figure 1 – Schematic representation of larvae path angles and grouped classes (Cl. 1, Cl. 2, Cl. 3, and Cl. 4) considered on the locomotor behavior assay. The angles of figure are not at scale.

2.6. Biochemical endpoints

Based on the results from FET test, embryos were exposed to one concentration with phenotypical effects (10 mg/L) and another without phenotypical effects (1 mg/L) of BNPs and VNPs. A surfactant control group (triton X-100 at 0.001%) was also included in the assay. Seven replicates of 15 embryos each were used per experimental condition, and embryos were kept in Petri dishes at 26 ± 1°C. After 96 h exposure, the embryos were frozen in liquid nitrogen and stored at -80°C, until further analyses.

Samples were homogenized in ultrapure water, on ice, using a sonic homogenizer (Sonifier 250, Branson sonicator). The homogenates were divided for the biochemical analyses: total glutathione (TG) content, glutathione S-transferase (GST) activity, cholinesterase (ChE) activity, protein quantification and energy budgets. Phosphate buffer (0.2 M; pH 7.4) was added to the homogenates aliquots reserved for TG, GST and ChE analyses. Then, the aliquots were centrifuged (10 000 g; 20 min; 4°C) to obtain the post-mitochondrial supernatant. A Labsystem Multiskan EX microplate reader was used for the biochemical determinations.

Biochemical markers were selected based on, in general, the available information about the mechanisms of toxicity of NPs. We selected biomarkers of the antioxidant system response since one of the most accepted mechanism of toxicity for NPs is the induction of ROS, leading to oxidative stress and/or damage (Mendoza and Brown, 2019). To understand if behavioral alterations are related with the neurotransmission system, the assessment of the activity of ChE was considered. Moreover, previous studies with zebrafish have shown the inhibition of this enzyme after the exposure to metals (Richetti et al., 2011). Energy metabolism biomarkers were studied because they may be related to embryonic morphological alterations. In addition, previous studies have shown the potential of NPs to affect energy metabolic pathways (Wang et al., 2019).

2.6.1. Total glutathione content

TG content was determined based on absorbance at 412 nm following the method of Tietze (1969). The formula of Beer-Lambert was applied to quantify TG content expressed as pmol/min/mg protein, using $\varepsilon=14.1x10^{3}$ $M^{-1}.cm^{-1}$.

2.6.2. Glutathione S-transferase activity

GST activity was measured photometrically at 340 nm following the method of Habig et al. (1974). The formula of Beer-Lambert was applied to quantify GST activity expressed as nmol/min/mg protein, using $\varepsilon=9.6x10^{3}$ $M^{-1}.cm^{-1}$.

2.6.3. Cholinesterase activity

The measurement of ChE activity was done following the protocol defined by Ellman et al. (1961), and adapted to a microplate format by Guilhermino et al. (1996). The absorbance was read at 414 nm. The formula of Beer-Lambert was applied to quantify the ChE activity expressed as nmol/min/mg protein, using $\varepsilon=13.6x10^{3}$ $M^{-1}.cm^{-1}$.

2.6.4. Protein quantification

The quantification of the protein was done following the Bradford method (Bradford, 1976), adapted to 96-well plates, using bovine γ- globuline as the standard. The absorbance was read at 600 nm.

2.6.5. Energy reserve levels

Energy budgets were assessed following the method of De Coen and Janssen (1997), with slight modifications for microplate reading described by Rodrigues et al. (2015) and listed below.

For lipid level measurements, 500 µL of chloroform and 500 µl of methanol were added to each sample, followed by centrifugation (1000 x g; 5 min; 20ºC). Then 500 µL of sulphuric acid (H_2SO_4) were added to the organic phase of each sample and incubated at 200°C for 15 min. After cooling down to room temperature, 1500 µL of ultrapure water were added, and the absorbance

was measured at 375 nm. Tripalmitin was used as a lipid standard. For the carbohydrate and protein level evaluations, 100 µl of 15% trichloroacetic acid (TCA) were added to each sample, followed by an incubation for 10 min at -20°C. After centrifugation (1000 g; 10 min; 4°C), 200 µL of 5% phenol, and 800 µL of H_2SO_4 were added to the supernatant. Glucose was used as the standard, and the absorbance was read at 492 nm. For protein measurements, the pellets were resuspended in 500 µL of sodium hydroxide (NaOH), incubated (30 min; 60°C), and neutralized with 280 µL of hydrochloric acid (HCl). Bradford's method (Bradford, 1976) was used, and absorbance was measured after 30 min incubation in the microplate at 520 nm. For the lipid, carbohydrate, and protein level measurements, absorbance was read at 375, 492, and 600 nm, respectively. The corresponding enthalpy of combustion (39 500 mJ/g lipid, 17 500 mJ/g glycogen, and 24 000 mJ/g protein) was used for conversions into the respective energetic equivalent values that were expressed as mJ/embryo.

2.7. Data analysis

Graphics and statistical analyses were performed using the Sigma Plot 12.5 software package. The data from the FET experiments and locomotor behavior assay were statistically analyzed by considering each well as an independent replicate. Shapiro-Wilk and Levene's tests were done to assess the normality and homoscedasticity of data, respectively. Differences between control and surfactant control were assessed using a Student's t-test. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post hoc test was used to assess differences between surfactant control and treatments. When data failed the normality and/or homoscedasticity tests, a

non-parametric Kruskal-Wallis' test was performed. Significant differences were accepted for $p<0.05$.

3. Results

3.1. Characterization of the test nanomaterials

BNPs and VNPs in the working dispersions revealed mean hydrodynamic sizes of 150 and 100 nm, respectively, maintaining the mean sizes over the entire 96 h exposure period (Table S1, Supplementary Data). However, in the experimental media, the mean hydrodynamic size of both NPs (at 10 mg/L) increased when compared with their hydrodynamic sizes in the working dispersions (Table S1). ZP values were negative for both NPs (Table S1). At concentrations ≤1 mg/L, it was not possible to characterize the NPs due to the detection limits of the techniques used.

3.2. Quantification of boron and vanadium

Considering all NP treatments, the difference between the nominal and measured concentrations was more evident for vanadium (Table S2, Supplementary Data). Unexpectedly, very low vanadium concentrations were detected (<98% of the nominal concentrations), while boron was measured at higher than nominal levels, in particular, for the two highest nominal concentrations 1 (140%) and 10 mg/L (115%). Comparing 0 and 96 h, a concentration decrease for both elements (boron and vanadium) was found with increasing exposure time (Table S2, Supplementary Data).

3.3. Fish Embryo Acute Toxicity Test

There were no significant differences, considering all the endpoints assessed during the FET test, between the medium only control and surfactant control ($p>0.05$). Differences were assessed between the NP treatments and surfactant control. BNPs caused no effects on survival and hatching of embryos ($P=0.553$ and 0.548; Figures 2A and B). However, significant induction of malformations was detected at 10 mg/L ($p<0.001$; Figures 2C and S1). VNPs caused no effects on survival of embryos ($p=0.652$; Figure 2A). However, a delay in hatching and an induction of malformations were detected at 10 mg/L ($p=0.001$ and <0.001, respectively; Figures 2B-C and S1).

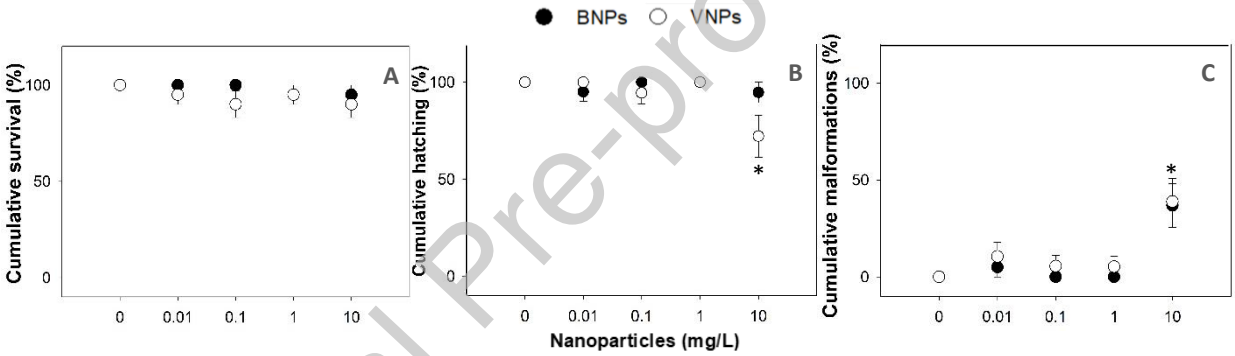


Figure 2 – Effects of 96 h exposure to boron (BNPs) and vanadium (VNPs) nanoparticles on zebrafish embryos in terms of: survival (**A**), hatching (**B**), and occurrence of malformations (**C**). Results are expressed as average values ± standard errors. (*****) Significant differences relative to surfactant control ($p<0.05$). Due to the overlap of some of the values between BNPs and VNPs experiments, only one symbol appears represented in the graph in a few cases.

3.4. Locomotor behavior assay

Zebrafish larvae typically present low levels of activity during light and increase their locomotor activity upon a sudden switch to darkness. This was observed in the current study; therefore, only the data obtained during the 6 min dark period is shown. Indeed, significant effects of NPs on larvae locomotion

348 were observed during the dark period (Figures 3, 4 and 5). There were no
349 significant differences ($p>0.05$) between the medium only control and surfactant
350 control for the assessed endpoints during the locomotor behavior assay, with
351 exception of zebrafish larvae path angle frequency in the class 4 angle
352 category. Therefore, differences were assessed between the NPs treatments
353 and surfactant control.

354 BNPs did not change the total distance swam by zebrafish larvae
355 ($p>0.05$; Figure 3A). However, 10 mg/L VNPs increased the total swimming
356 distance of larvae ($p=0.003$; Figure 3A). No effects of BNPs or VNPs were
357 detected on total swimming time of organisms ($p>0.05$; Figure 3B). However, a
358 decrease in time and distance swimming on external zone of well was detected
359 at all tested concentrations for both NPs (Figures 3C-D).

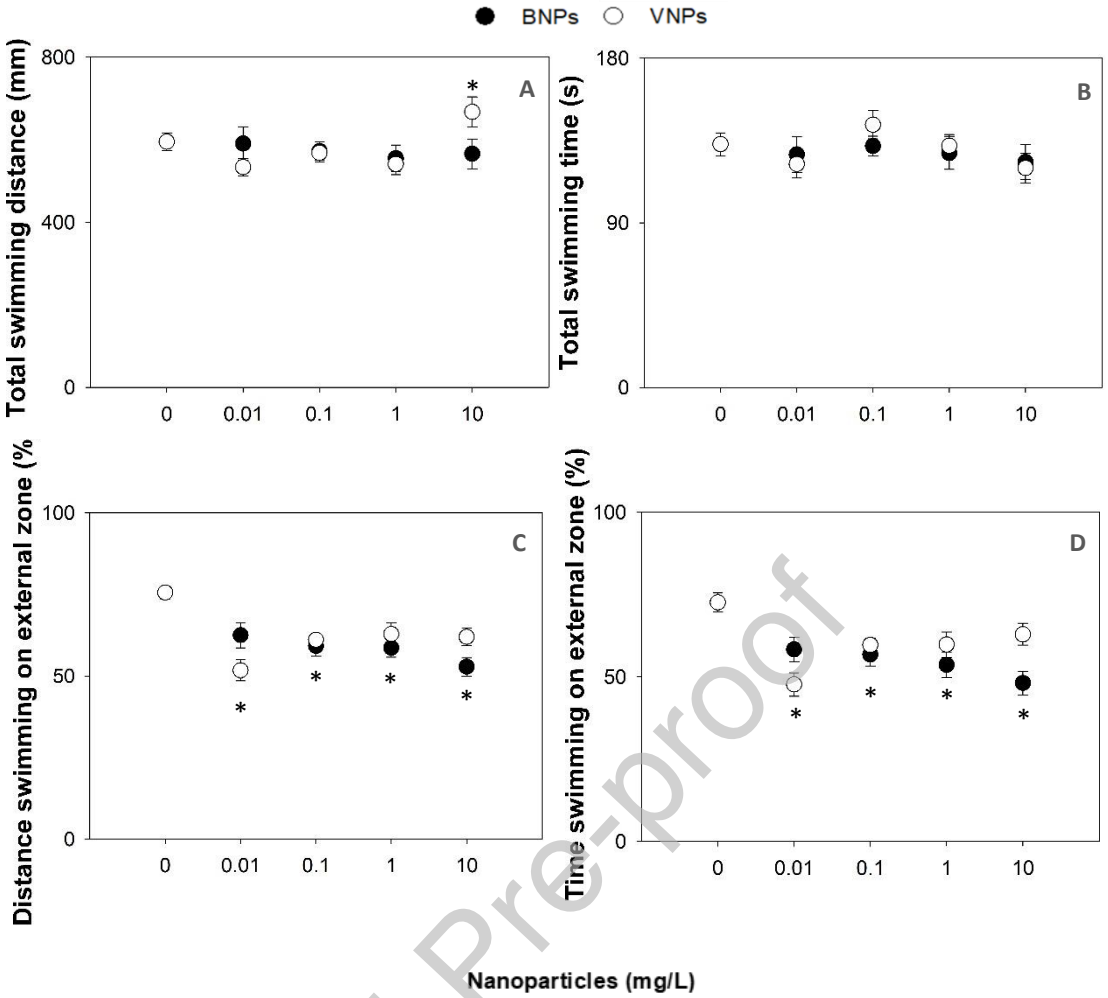


Figure 3 – Effects of 120 h exposure to boron (BNPs) and vanadium (VNPs) nanoparticles on zebrafish larvae in terms of: total swimming distance (**A**) and time (**B**); swimming distance (**C**) and time (**D**) in external zone. Results are expressed as average values ± standard errors. (*****) Significant differences relative to surfactant control ($p<0.05$).

BNPs, at 10 mg/L, increased the frequency of class 1 angles ($p=0.027$) and decreased the frequency of class 4 angles at 1 and 10 mg/L ($p=0.044$ and 0.005, respectively; Figure 4A). VNPs also increased the frequency of class 1 angles at 0.01and 10 mg/L ($p<0.001$) and the frequency of class 2 angles at 0.01 mg/L ($p=0.021$); Figure 4B). A decrease of class 4 angles frequency was also detected at 0.01 mg/L ($p=0.036$) as well as a decrease of class 3 angles frequency at 10 mg/L ($p<0.001$; Figure 4B).

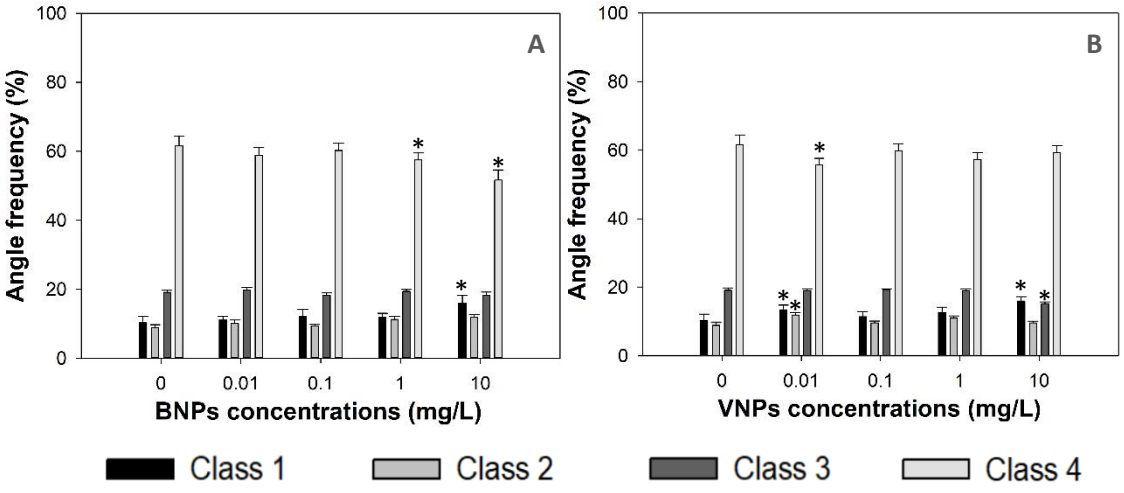
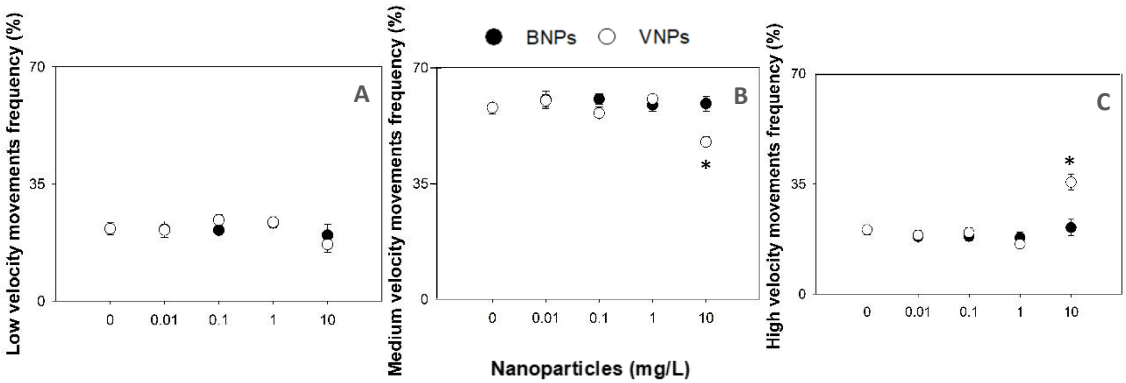


Figure 4 – Effects of 120 h exposure to boron (BNPs) (**A**) and vanadium (VNPs) (**B**) nanoparticles on zebrafish larvae path angle frequency. Class 1 includes big amplitude angles (90-180º), classes 2 and 3 include medium amplitude angles (30-90º and 10-30º, respectively), and class 4 includes small amplitude angles (0-10º). Results are expressed as average values ± standard errors. ***** Significant differences relative to surfactant control ($p<0.05$).

BNPs did not affect the frequency of the three types of movements considered ($p>0.05$) (Figure 5). However, 10 mg/L VNPs decreased medium ($p<0.001$) velocity movements and increased high ($p<0.001$) velocity movements of zebrafish larvae (Figure 5).



391

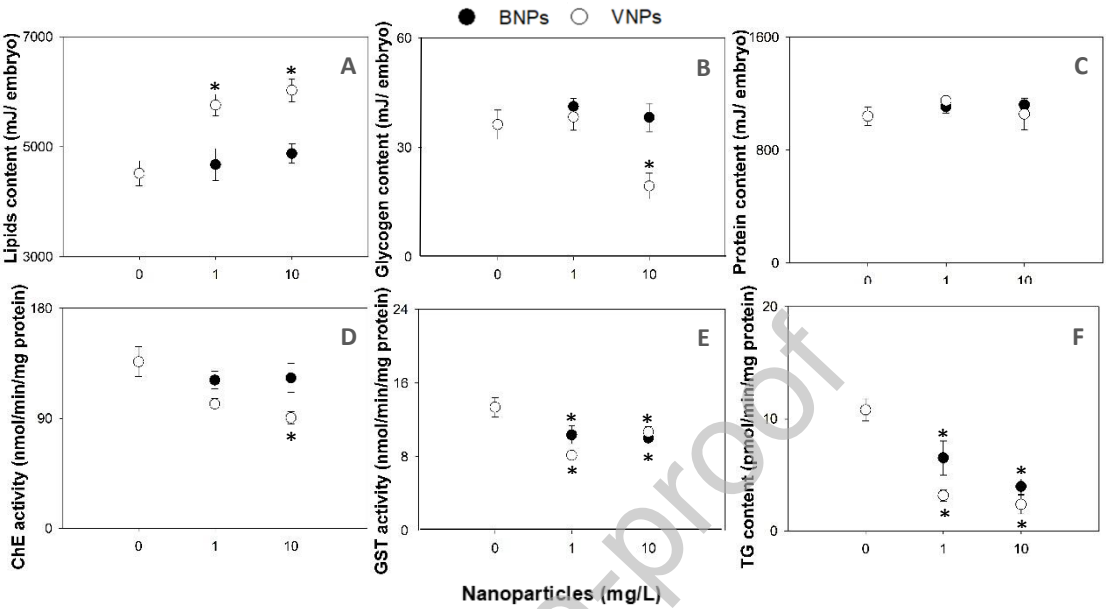
392 **Figure 5** – Effects of 120 h exposure to boron (BNPs) and vanadium (VNPs) nanoparticles on
393 the frequency of low (**A**), medium (**B**), and high (**C**) velocity movements of zebrafish larvae. Low
394 velocity includes movements below 8 mm/s; medium velocity for movements between 8; and
395 40 mm/s, and high velocity consists of movements above 40 mm/s. Results are expressed as
396 average values ± standard errors. (*****) Significant differences relative to surfactant control
397 ($p<0.05$). Due to the overlap of some of the values between BNPs and VNPs experiments, only
398 one symbol appears represented in the graph in a few cases.

399 **3.4. Biochemical markers assessment**

400 There were no significant differences ($p>0.05$) between the medium only
401 control and surfactant control for the assessed biochemical markers.
402 Differences were assessed between the NPs treatments (1 and 10 mg/L NPs)
403 and surfactant control.

404 BNPs caused no effects on lipid, carbohydrate and protein contents or ChE
405 activities ($p>0.05$; Figures 6A-D). Only a decrease in TG content ($p=0.028$ and
406 <0.001) and GST activity ($p=0.044$ and 0.025) was detected at both
407 concentrations of BNPs (Figures 6E-F). In contrast, VNPs affected almost all
408 biochemical markers analyzed, except for protein content ($p>0.05$; Figure 6C).
409 Both concentrations of VNPs increased the lipid content ($p=0.001$ and <0.001,
410 respectively; Figure 6A). At 10 mg/L VNPs, a decrease in carbohydrate content

411 was detected (p=0.009; Figure 6B). ChE activity decreased at 10 mg/L VNPs
412 (p=0.005; Figure 6D). Both concentrations of VNPs decreased TG content
413 (p<0.001; Figure 6F) and GST activity (p<0.001 and p=0.028; Figure 6E).



414

415 **Figure 6** – Effects of 96 h exposure to boron (BNPs) and vanadium (VNPs) nanoparticles on
416 zebrafish larvae in terms of: lipid (**A**), carbohydrate (**B**), and protein (**C**) contents; cholinesterase
417 (ChE) (**D**) and glutathione S-transferase (GST) (**E**) activities; and total glutathione (TG) content
418 (**F**). Results are expressed as average values ± standard errors. (*****) Significant differences
419 relative to surfactant control (p<0.05).

420 **4. Discussion**

421 Dissimilar effects, i.e. phenotypical, biochemical and behavioral were
422 found on the development of zebrafish embryos when exposed to BNPs or
423 VNPs. Both NPs were not lethal at concentrations ≤10 mg/L. BNPs affected few
424 biological endpoints. At the organism level, BNPs induced larvae malformations
425 and impaired their swimming behavior, which was reflected by erratic swimming
426 and swimming pattern alteration. At the biochemical level, a drop in the content
427 of glutathione (substrate and enzyme) occurred showing an imbalance of the

antioxidant system. In addition to the organism level effects found in zebrafish larvae exposed to BNPs, VNPs also caused delayed hatching and hyperactivity. Furthermore, ChE activity decreased, which may be associated with the observed locomotion impairment. Similarly to BNPs, VNPs also reduced the levels and activity of antioxidant substances (linked to glutathione). Energetic-spending functions were also impaired by VNPs as demonstrated by the increase in lipid content and a decrease in carbohydrate content. The inhibition of lipid depletion in zebrafish larvae caused by VNPs may be related to the occurrence of yolk sac edemas. The zebrafish responses assessed at different hierarchical levels, i.e. from the apical to the biochemical, seem to be interconnected and dependent of the NPs nature, size and concentration.

The interaction of NPs with the environmental media may affect their initial physicochemical properties, and thus, their fate and ecotoxicity (Tourinho et al., 2012). No alterations in surface charge (ZP) were found between 0 and 96 h for both NPs, (-30 mV), hence colloidal stability was expected in the experimental medium (Jiang et al., 2009). A similar stability in terms of ZP values during 96 h exposure was also reported by Liu et al. (2019) for Ag NPs in zebrafish medium. Although both NPs maintained their hydrodynamic sizes during the 96 h exposure time in the working dispersions, there was a size increase relative to working dispersions when NPs were in experimental medium, suggesting aggregation/agglomeration may have occurred. Changes of pH and ionic strength or the presence of biomolecules, particularly proteins, can modify the physiochemical properties of NPs (e. g. size and surface charge), leading to the loss of colloidal stability and formation of agglomerates/aggregates (Halamoda-Kenzaoui et al., 2017). These

453 agglomeration/aggregation processes may result in increased NPs
454 hydrodynamic sizes, as observed in our study (at the experimental media), and
455 may affect the NPs bioavailability, uptake, bioaccumulation, and toxicity
456 (Albanese and Chan, 2011; Corsi et al., 2020). In zebrafish medium, previous
457 studies also showed the aggregation/agglomeration of other NPs, such as
458 silicon dioxide (SiO_2), Ag, and CuO (Liu et al., 2019; Thit et al., 2017; Zhu et al.,
459 2019). Liu et al. (2019) described that zebrafish medium contains
460 chloride/sulfate anions and divalent cations, which may induce agglomeration,
461 regardless of the primary size of the particles, reducing the surface area and
462 dissolution of NPs. A single study that characterized BNP suspensions also
463 showed a fast aggregation of particles (increased sizes) after 48 h of exposure
464 to *Daphnia magna* (Strigul et al., 2009). Furthermore, BNPs presented a
465 hydrodynamic size greater than that of VNPs at 0 h (361 versus 181 nm) and at
466 96 h (527 versus 290 nm). Both NPs suffered processes of
467 aggregation/agglomeration in the test medium; however, the BNP
468 aggregates/agglomerates were bigger than VNP aggregates/agglomerates. The
469 detected difference in terms of NP aggregation/agglomeration processes (BNPs
470 aggregates/agglomerates > VNPs aggregates/agglomerates) may be explained
471 by their differences in terms of the initial hydrodynamic size of theNPs (in
472 working dispersion, BNPs: around 150 nm; VNPs: around 100 nm) and ZP (in
473 working dispersion, ZP BNPs more negative than ZP VNPs). Hence, the
474 presence of agglomerates/aggregates may affect the degree of uptake and
475 bioavailability and may reduce the dissolution of NPs, leading to differential
476 toxicity (Albanese and Chan, 2011; Corsi et al., 2020).

Measured concentrations of vanadium in exposure medium were very low, compared with the nominal concentrations. The obtained results may be explained by the agglomeration/aggregation of VNPs and subsequent sedimentation, leading to less particles in suspension in the aqueous media, as already found for other metallic NPs (Barreto et al., 2019). The final test concentrations were obtained from serial dilutions of the working dispersion. Since the VNPs aggregated/agglomerated, this affected the accuracy of the dilution and consequently, the nominal concentrations were not reflected in the measured concentrations. BNPs also suffered agglomeration/aggregation processes in the zebrafish experimental medium and the hydrodynamic sizes of the resultant agglomerates/aggregates were bigger than aggregates/agglomerates of VNPs. Therefore, it was expected that the measured concentrations of boron would be even less, compared with the nominal concentrations. However, the measured concentrations of boron were higher (at the two highest concentrations) than expected. This may be due to the presence of boron in the filtered tap water since the conventional water treatment processes can be inefficient in its removal (Bhagyaraj et al., 2021). Furthermore, boron is essential for zebrafish development (Rowe and Eckhert, 1999) naturally occurring in zebrafish medium. Therefore, its “basal” occurrence may explain (at least partially) the data obtained in terms of boron quantification, including the presence of boron in the controls (where no BNPs where added). Nevertheless, the higher values found in the two highest concentrations may not be explained only based on the “basal” levels in the zebrafish water. Higher aggregation/agglomeration occurred in the working dispersion and experimental media, which may have resulted in pipetting

greater NP mass from the bottom of the dispersion (due to NPs sedimentation) during preparation of the two highest test concentrations (10 and 1 mg/L). Physicochemical characteristics and the intrinsic processes (such as aggregation/agglomeration) of each element that occurred in the zebrafish water, may explain the differences found in terms of boron and vanadium quantification in the experimental media.

Since zebrafish chorion pore size is 600-700 nm, the used NPs (80-100 nm were expected to penetrate the chorion, even when agglomerated/aggregated (<527 nm). However, at the tested concentrations, exposure to NPs for 96h did not induce mortality to zebrafish embryos. Few studies reported no effects on survival of zebrafish embryos after exposure to similar concentrations of other NPs, e. g. 10 mg/L of TiO_2 (25-40 nm) (Tang et al., 2019); 0.1, 1, and 10 mg/L of Ag (20-40 nm, 10 and 100 nm) (González et al., 2018; Liu et al., 2019); and 12.5 mg/L of SiO_2 (25 and 115 nm) (Vranic et al., 2019; Zhu et al., 2019). However, the single ecotoxicity study previously conducted with BNPs reported a lethal concentration at which 50% of the test population died (LC_{50}) at 6.7 mg/L for *D. magna* after 48 h of exposure, suggesting higher sensitivity of this species compared with zebrafish (Strigul et al., 2009). The different results may also be explained by the dissimilar characteristics of BNPs (mean diameter: 10-20 nm in *D. magna* study versus 80-100 nm in the present study). NP toxicity is dependent on a variety of factors, namely size, agglomeration state, dissolution rate, concentration, and coating (Aksakal and Ciltas, 2019).

Despite being non-lethal, 10 mg/L of both NPs significantly induced malformations (e. g. spinal malformation, yolk-sac, and pericardial edemas) in

527 zebrafish embryos. Similar malformations were induced in zebrafish embryos by
528 other NPs: ≥1 mg/L of zirconia (ZrO) (15-20 nm) (Karthiga et al., 2019); 1.925
529 mg/L of Ag (4 nm) (Qiang et al., 2020); ≥50 mg/L of SiO_2 (15 and 30 nm) (Zhu
530 et al., 2019); and ≥0.5 mg/L of CuO (≤50 nm) (Aksakal and Ciltas, 2019). Boron
531 is considered an essential element for zebrafish development (Rowe and
532 Eckhert, 1999), with a safe range of 0.0022 to 99.5 mg/L (Rowe et al., 1998).
533 The teratogenic effects of BNPs reported in the present study show that boron
534 might be more toxic in nanoparticle form than in its ionic/elemental form. For
535 vanadium compounds, such as sodium metavanadate, vanadium pentoxide,
536 and oxovanadium sulfate, previous studies have reported the ability to induce
537 teratogenic effects (at 10 mg/L), namely yolk-sac edemas and pericardial
538 edemas in zebrafish embryos after 96 h exposure (Bittencourt et al., 2018).
539 Inorganic vanadium compounds were also shown to be toxic to mammals,
540 causing neurobehavioral injuries, impairment in development and reproduction
541 as well as morphological and functional lesions in organs (Ghosh et al., 2015).

542 In the present study, in addition to inducing malformations, 10 mg/L
543 VNPs also delayed hatching of embryos. Other authors also reported impaired
544 hatching at ≥0.5 mg/L of CuO (≤50 nm) (Aksakal and Ciltas, 2019), ≥0.1 mg/L
545 ZnO (10-12 nm) (Morgalev et al., 2018), and ≥1 mg/L of ZrO (15-20 nm) NPs
546 (Karthiga et al., 2019). Delaying hatching may be due to blockage of the
547 secretory function of hatching gland cells, inactivation of chorionase (hatching
548 enzyme), suppression of embryogenesis, and an impaired ability of the larvae to
549 break the chorion due to the presence of malformations (Aksakal and Ciltas,
550 2019; Morgalev et al., 2018).

Exposure of zebrafish larvae to both NPs resulted in differential effects on swimming behavior in the light:dark assay. Larvae exposed to 10 mg/L VNPs increased the total distance moved, particularly the frequency of high velocity movements, suggesting hyperactivity. Moreover, the trend to decrease straightforward movements (as low amplitude angles – class 4, at 0.01 mg/L VNPs) and the increase in zigzag movements or movements with changes of direction (as large amplitude angles – class 1, at 0.01 and 10 mg/L), suggests erratic swimming behavior (Almeida et al., 2019). Additionally, with increasing VNPs concentrations, larvae spent less time in the external area of the well, which suggests an alteration in the swimming pattern probably related to the zigzagging behavior observed. These results demonstrate that VNPs may disrupt locomotor behavior at concentrations that caused no mortality (although at relatively high and not environmentally relevant concentrations), which is consistent with the finding that behavior is among the most sensitive endpoints in zebrafish toxicity screening (González et al., 2018). On the other hand, BNPs did not affect the total distance swam by larvae or the frequency of the different types of movements. BNPs (as VNPs) induced erratic swimming (zigzagging behavior) in zebrafish larvae, with decrease of low amplitude angles at 1 and 10 mg/L, and accompanied by an increase of large amplitude angles at 10 mg/L as well as an alteration in the swimming pattern (similarly to VNPs). González et al. (2018) reported hyperactivity of zebrafish larvae after exposure to 0.3, 1, and 3 mg/L of Ag NPs (20-40 nm).. However, Powers et al., (2011) concluded that smaller Ag NPs decreased larvae locomotor activity whereas the larger ones caused hyperactivity, i.e. a size dependent effect. Chen et al. (2011) obtained different effects depending on NP concentrations: at low levels of TiO_2 NPs, a

576 decrease in the velocity/activity levels and an increase in burst velocity were
577 detected, but the effects were not significant at the higher tested concentrations.
578 Meanwhile Thit et al. (2017) reported no effect for 0.5 to 200 µM of CuO NPs (6
579 nm) on the total distance swam by zebrafish larvae. The effects on behavior
580 described by this study, and by previous studies with zebrafish larvae, suggest
581 that behavior alterations are different depending on the NPs used, which we
582 hypothesize to be a function of the type, size and concentration of the NPs.

583 Lipids are the first energy source mobilized when organisms are
584 exposed to contaminants, and along with carbohydrates, they are quickly
585 mobilized to supply a sudden energy demand. Proteins are the last choice of
586 energy source, being mobilized only under severe conditions (Abe et al., 2018).
587 Contaminants might be able to impair the total energy available that initiates
588 compensatory adjustments in the energy metabolism of organisms to maintain
589 physiological homeostasis (Abe et al., 2018). In the present study, VNP
590 exposed larvae exhibited higher lipid content and lower carbohydrate content,
591 while protein content remained similar to control larvae. This may be caused by
592 an inhibition of lipid depletion in zebrafish larvae triggered by VNP exposure,
593 which may be related to the observation of embryos exhibiting yolk sac edemas
594 caused by inhibition of yolk sac resorption, suggesting that energetic-spending
595 functions might be impaired (Abe et al., 2018). Moreover, these effects on
596 energy metabolism may compromise other physiological functions, such as
597 growth, reproduction, development, and locomotor activity. Verma et al. (2018)
598 showed accumulation of neutral lipids in zebrafish embryos exposed to 50 and
599 250 mg/L of TiO_2 NPs (85 nm). In contrast, BNP exposure did not affect energy
600 reserves contents. However, exposed embryos also exhibited yolk sac edemas.

These edemas were not caused by distended yolk content resulting from inhibition of lipid depletion since the levels of lipids remained similar to control larvae. However, yolk sac edemas may also be caused by impaired maintenance of the osmotic gradient resulting in excessive water uptake (Sant and Timme-Laragy, 2018). Although both NPs caused yolk sac edemas, different mode of actions may be involved regarding the observed effect.

GST is a family of enzymes involved in phase II of the detoxification process, while TG is very important in non-enzymatic antioxidant defense through direct interaction of its sulfhydryl (SH) group with ROS (Almeida et al., 2019). Decreased TG content and GST activity by both NPs may be interpreted as a sign of cytotoxicity due to an over-production of ROS, suggesting an impairment of conjugation and antioxidant processes that ultimately can lead to oxidative damage (Meireles et al., 2018; Zhu et al., 2019). However, levels of ROS needed to be quantified in order to confirm this hypothesis. In vivo and in vitro studies applying different biological matrixes have demonstrated oxidative stress caused by VNPs. Wistar rats exposed to VO_2 NPs induced higher levels of malondialdehyde and reduced glutathione (Kulkarni et al., 2014). In lung cell line A549 VNP exposure was found to cause elevated ROS generation (membrane damage and apoptosis)(Xi et al., 2019). Moreover, ROS generation was reported as the toxic mechanism of VO_2 particles (Fickl et al., 2006; Kulkarni et al., 2014; Wörle-Knirsch et al., 2007; Xi et al., 2019) and it was associated with the dissolution of VO_2 from NPs, i.e., could be explained by both oxidation state and size (Wörle-Knirsch et al., 2007; Xi et al., 2019). Regarding BNPs, a study has shown that boron nitride (BN) NPs increased oxidative stress levels in *Caenorhabditis elegans* by promoting ROS production

626 (Wang et al., 2017). Daglıoglu and Ozturk (2018) also concluded that
627 considering ROS analysis, boron particles induced oxidative stress on the algae
628 *Chodatodesmus mucronulatus.*Tang et al. (2019) found decreased superoxide
629 dismutase (SOD), catalase (CAT), and GST activities in adult zebrafish after
630 exposure to 100 mg/L of TiO_2 NPs (25-40 nm). Similarly, 100 mg/L of SiO_2 NPs
631 (15 nm) also induced oxidative damage in zebrafish larvae, with increased ROS
632 and malondialdehyde (MDA) content, decreased SOD activity and reduced
633 glutathione (GSH) content (Zhu et al., 2019).

634 ChE is essential for the normal function of the zebrafish nervous system
635 and any functional disturbance in this enzyme may cause adverse effects on
636 the locomotor behavior of zebrafish larvae, such as erratic movements and
637 hyperactivity (Gaaied et al., 2020). VNPs inhibited ChE activity at 10 mg/L. This
638 inhibition may result in the accumulation of acetylcholine in the synaptic cleft
639 and leads to a disruption of nervous system function (Almeida et al., 2019).
640 Hence, VNPs can alter the cholinergic system by affecting ChE activity, which
641 may be involved in the locomotion impairment observed for the exposed larvae,
642 specifically the hyperactivity observed. In contrast, the locomotor behavior
643 alterations detected in larvae exposed to BNPs seem not be related to
644 cholinergic damage (specifically ChE activity) since the activity of this enzyme
645 was not altered at any tested concentration of BNPs.

646 The tested NPs induced dissimilar effects to zebrafish embryos, with
647 VNPs affecting more endpoints than BNPs at the same concentrations,
648 indicating the greater toxicity of VNPs. The different chemical nature of these
649 nanomaterials may imply differential modes of action in zebrafish embryonic
650 development and larvae locomotion, emphasising the importance of evaluating

651 the effects of different types of NPs. On the other hand, despite both NPs
652 undergoing aggregation/agglomeration in zebrafish medium, BNPs
653 aggregates/agglomerates were bigger than VNPs aggregates/agglomerates.
654 This difference in terms of aggregates/agglomerates sizes may also explain the
655 lower toxicity of BNPs to zebrafish embryos, compared with VNPs. When
656 aggregates/agglomerates become too large for direct transport across the cell
657 membrane, uptake may be reduced and less effects to the organisms are
658 expected (Vale et al., 2016).

659 Overall, the current study demonstrated that BNPs and VNPs affects
660 zebrafish embryos at relatively great concentrations, reinforcing the importance
661 of NPs environmental risk assessment. Despite being non-lethal, both tested
662 NPs induced significant effects on several endpoints, from the biochemical to
663 the organism level, highlighting the relevance of a multi-endpoint
664 ecotoxicological evaluation, at different levels of biological organization, to
665 screen the potential toxic effects of NPs. Moreover, the effects of the tested
666 NPs reported in this study occurred at concentrations that were greater than
667 those commonly found in the environment, making it difficult to elaborate any
668 predictions regarding ecological effects, resulting in the need for studies with
669 lower environmentally relevant concentrations (e. g. < 0.01 mg/L), to
670 understand if the toxic effects are maintained, especially at the biochemical
671 level. Additionally, further studies evaluating other parameters, such as those
672 involved in oxidative stress/damage (e. g. lipid peroxidation levels), other
673 antioxidant system, as enzymes (e. g. CAT and SOD activities) and substrates
674 (e. g. metallothioneins), genotoxicity, as well as, gene expression (e. g. genes

related with oxidative stress and neurotransmission) are needed for a more complete understanding about the modes of action of BNPs and VNPs.

Author contributions

Joana Santos: Conceptualization, Methodology, Formal Analysis, Investigation, Writing—Original Draft Preparation, Writing—Review and Editing. Ângela Barreto: Conceptualization, Methodology, Writing—Review and Editing. Célia Almeida: Formal Analysis, Investigation. Cátia Azevedo: Formal Analysis, Investigation. Inês Domingues: Formal Analysis, Resources, Writing—Review and Editing. Mónica, J. B. Amorim: Resources, Writing—Review and Editing. Vera L. Maria: Conceptualization, Methodology, Resources, Writing—Review and Editing, Project Administration, Funding Acquisition.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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