Acute Adverse Effects of Metallic Nanomaterials on Cardiac and Behavioral Changes in *Daphnia Magna*

Jihoon Park

National Institute of Chemical Safety

Chang Gyun Park

KIST Europe

Baeckkyoung Sung

KIST Europe

Yong Oh Lee

KIST Europe

Chang Seon Ryu

KIST Europe

Ja Young Park (✉ pjy0404@gmail.com)

KIST Europe

Young Jun Kim

KIST Europe

Research Article

Keywords: nanoparticle, toxicity, water flee, heart rate, swimming speed, oxidative stress

Posted Date: February 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-208731/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Environments on February 10th, 2022. See the published version at <https://doi.org/10.3390/environments9020026>.

Abstract

Nanomaterials are widely believed to induce toxic effects on organisms by evoking oxidative stress. In this study, we evaluated the toxic effects of nanomaterials on cardiac and behavioral changes in *Daphnia magna* under varying exposure conditions. Titanium dioxide nanoparticles (TiO₂ NPs), silver nanoparticles (AgNPs), and silver nitrate (AgNO₃) were selected for the acute toxicity tests. The adverse effects of the substances on the neonates including heart rate, swimming speed, and oxidative stress, were measured. The heart rate level decreased as the concentration of NPs and silver ions (Ag⁺) increased. The average swimming speed was measured to be approximately 15 mm/min for the control group. The swimming speed generally increased for longer exposure to both NPs, although it reached a plateau at the lowest concentration of AgNPs. A similar but less clear trend was observed for Ag⁺. For all substances, the overall swimming speed exhibited no correlation or weak negative correlations with the exposure concentration. The oxidative stress levels increased after exposure compared to the control group. We conclude that aquatic nanotoxicity tests should consider multilevel physicochemical, physiological, and behavioral parameters for the official guidelines to quantify more robust adverse outcomes.

Introduction

Nanomaterials are used in a wide range of sectors due to their specific physicochemical properties compared to their bulk materials which have the same chemical composition¹⁻³. To ensure safe use, it is important to identify and manage the effects of exposure to nanomaterials on human and environmental health more effectively. In particular, the aquatic phase is considered a key starting point of potential entry and diffusion into the environment for understanding environmental fate and behavior. The aquatic phase creates connections with other environmental compartments such as soil, sediment, and air⁴.

Several studies indicate that nanomaterials are toxic to aquatic organisms such as fish and crustaceans⁵⁻⁷. Of these, fishes have a direct impact on human health, but it is difficult to obtain information on the exact lethal concentration of nanomaterials from fish. Therefore, the impact of nanomaterials on non-target organisms will play an important role⁸. Aquatic invertebrates are a very large group of animals and some have over 1000 different types of species. Among them, arthropods are the largest invertebrate, consisting of the two largest groups: insects and crustaceans⁹. Crustaceans are the most important group of invertebrates, of which *Daphnia magna* is commonly used to assess the risk of nanomaterials¹⁰. Algae and crustaceans exposed to nanomaterials can poison and cause death to aquatic organisms such as fish through the food chain^{5, 11}.

Nanomaterials are generally known to have higher toxicity than bulk materials. It has been reported from *in vitro* studies that nanomaterials can induce biological effects such as cell inflammation and apoptosis by oxidative stress¹²⁻¹⁵. The preliminary factors causing toxicity are size, shape, and surface area/charge¹⁶. Nanomaterials are more reactive due to their large surface area, and easy penetration into

cells^{16, 17}. Depending on the surface properties, the nanomaterials may exhibit enhanced interactions with the cell membranes^{18, 19}.

Here, we focus on two representative types of metallic nanoparticles, titanium dioxide (TiO₂) and silver (Ag) nanoparticles (NPs). TiO₂ NPs are generally known to be non-toxic and can be applied in various ways such as through cosmetics and catalysts^{20, 21}. AgNPs are also used in a wide range of consumer products as antibacterial agents due to their high cytotoxicity^{22–24}. One of the most fundamental sources of AgNP toxicity has been attributed to the release of silver ions (Ag⁺) in tissue environments.

Daphnia magna is a standard organism for ecotoxicity test using toxic chemicals in the standardized protocols suggested by the Organization for Economic Cooperation and Development (OECD), International Organization for Standardization (ISO), and the United States Environmental Protection Agency (USEPA)^{25, 26}. As an organism in the bottom stage of the food chain, *D. magna* plays a key role in aquatic ecosystems since it is sensitive to environmental stresses, and population change can ultimately affect the population of upper predators^{25, 27}. Previous studies have reported the biological effects of hazardous chemicals on physiological factors such as cardiac and behavioral characteristics in *D. magna*^{28–32}. Fekete-Kertész et al. (2017) evaluated the change in heart rate using *D. magna* and found that heart rate was influenced by several factors; chemical exposure level (triclosan), test medium, organism age, and exposure time³³. Chung et al. (2016) also reported that the heart rate and swimming distance were linearly changed by TiO₂ nanoparticle concentration³⁴. In particular, they adopted a video tracking method which has been recently used to evaluate the behavioral characteristics of organisms such as zebrafish and *D. magna*. Therefore, we aimed to evaluate the toxic effects on the physiological factors of *D. magna* using nanomaterials and to identify the relationships between the factors according to the exposure conditions in this study.

Results

Effects of nanomaterials on the heart rate

Immobilization and heart rate were measured after acute exposure. The acute immobilization test was performed in accordance with OECD guidelines 202. A median lethal concentration (LC₅₀) value of TiO₂ in *D. magna* after 48 hours was determined to be more than 100 mg/ml and the LC₅₀ values of AgNPs and Ag⁺ were 0.0134 and 0.0016 µg/ml, respectively (Fig. 1).

The heart rate decreased as the concentration of all substances (TiO₂ NPs, AgNPs, Ag⁺) increased. The heart rate of the control group was observed to 364 ± 28 BPM (beats per minute). At the lowest concentration level of each substance after exposure for 3 and 48 hours (355–385 BPM), the heart rate was not significantly different from that of the control level, but it decreased by approximately 7.3% (TiO₂, 345 ± 17 BPM), 4.3% (AgNP, 347 ± 26 BPM), and 15.0% (Ag⁺, 302 ± 15 BPM) at the highest concentration level after exposure for 3 hours, respectively (Fig. 2A). After 48 hours of exposure, the heart rate recovered

at the lowest and medium concentration levels compared to the control level. At the lowest concentration of each substance, the heart rate was 378 ± 25 BPM for TiO₂ NPs, 385 ± 14 BPM for AgNPs, and 375 ± 17 BPM for Ag⁺, but it gradually decreased with increasing concentration level for all substances (Fig. 2B). The reduction rate with a higher concentration was relatively lower than that after 3 hours of exposure (TiO₂ NPs, 5.8%; AgNPs, 2.4%; Ag⁺, 3.3%).

Effects on the behavioral performance

Behavioral performance was measured as the swimming speed, which is an averaged moving distance per minute and the speed level of the control group was approximately 15 mm/min. Real-time tracking paths were recorded according to the instantaneous swimming speed as shown in Fig. 3 with examples of 10-minute exposure. The observed behavioral change upon substance exposure was limited to locomotive movements. The swimming speed generally increased for longer exposure to both NPs, although it reached a plateau at the lowest concentration of AgNPs. A similar but less clear trend was observed for Ag⁺. The swimming behavior appeared to be more activated at the lowest concentration level of each substance compared to higher concentration levels. For the neonates that were exposed to 0.1 µg/mL TiO₂ NPs, the swimming speed increased proportionally with the exposure duration (from 8.2 ± 0.9 mm/min for 3 hours to 21.6 ± 2.8 mm/min for 48 hours). In addition, the swimming speed increased with increasing exposure duration, although the swimming speed was lower at higher concentrations (1.0 and 10 µg/mL) than at the lowest level (0.1 µg/mL) (Fig. 4A). In the case of AgNPs, the swimming speed showed irregular patterns by the concentration level and exposure duration. It significantly increased up to the 2-hour exposure duration from 7.4 to 17.2 mm/min, and decreased slightly after 24-hour exposure at 0.0001 µg/mL. It continuously increased in proportion to the exposure duration at 0.001 µg/mL from 6.7 to 16.0 mm/min, but this trend was weakened at the highest concentration (Fig. 4B). At the lowest concentration of Ag⁺ (0.00001 µg/mL), the speed increased proportionally up to 24 hours of exposure (from 7.0 ± 0.8 mm/min for 3 hours to 21.7 ± 4.1 mm/min for 24 hours). There was no obvious pattern at higher concentration levels, but the speed peaked for the neonates exposed for 24 hours (Fig. 4C). Real-time temporal variations in swimming speed for each material are available in the supplementary information (Fig. A1-A3).

Quantification of oxidative stress

Reactive oxygen stress (ROS) levels were measured by 2,7-dichlorofluorescein diacetate (DCFDA) assay. Figures 5 & 6 show the results of ROS measurements in *D. magna*. All three materials exhibited stronger intensities than the control group after 3 hours of exposure. The error bars in Fig. 6 represent the arithmetic mean and standard deviation ($n = 3$, mean $\pm$ SD). In particular, the ROS intensity of TiO₂ significantly increased in proportion to the concentration, but the AgNPs and their ions showed concentration-dependent tendencies and then decreased at the highest concentration (0.01 µg/mL for AgNPs and 0.001 µg/mL for Ag⁺). The results indicate that exposure to all substances for 3 hours significantly affected ROS levels, while exposure for 48 hours decreased ROS levels at a higher

concentration, which is likely due to the alteration of the effective concentration after 48 hours of exposure.

Discussion

The toxicity test using *D. magna* is well documented in the OECD guidelines 202 and it is determined by immobilization at 24 and 48 hours after exposure to nanoparticles. In most cases, it is difficult to determine accurate toxic effects based on the method because the data varies depending on the different groups. Considering this limitation, we tried to test the acute toxicity on *D. magna* using influential indices including immobilization, heart rate, swimming performance and ROS level.

First, we determined the LC₅₀ values for each material as the first step in the exposure test using organisms. The LC₅₀ value of TiO₂ in *D. magna* after 48 hours was determined to be more than 100 mg/ml and the values of AgNPs and Ag⁺ were 0.0134 and 0.0016 µg/ml, respectively. Ribeiro et al. (2014) determined the LC₅₀ of AgNPs to be 11.02 µg/L after 48 hours of exposure, and 1.05 µg/L for silver nitrate (AgNO₃) using *D. magna*³⁵. Shen et al. (2015) also calculated LC₅₀ values of 0.58–2.51 µg/L for AgNO₃, which were lower than our results³⁶. The results occur depending on the strain and water physicochemical parameters, which means that even if we measured the LC₅₀ considering experimental conditions, the outcomes might be different between laboratories.

The change in heart rate as an index of toxic effects on *D. magna* is widely used in acute toxicity tests. The heart rate was measured repeatedly using more than 10 neonates exposed to the materials and a cross check for all measurements was also performed to minimize bias by observers. The results showed that the heart rate decreased with increasing concentration for all substances. It was possible to obtain a similar pattern even for 3-hour exposure with the OECD guidelines (48-hour exposure)³⁷. Thus, we confirmed that an exposure duration of 3 hours was sufficient to test acute toxicity. In addition, TiO₂ NPs considered to be non-toxic, also reduced the heart rate by increasing the exposure level. This showed that TiO₂ NPs affect the heart rate of *D. magna*, similar to other toxic NPs (e.g., AgNPs), but it is limited to conclude that TiO₂ NPs influence the immobilization (behavioral performances).

Locomotion-based behavior is a highly sensitive index for identifying toxic effects of chemicals^{28, 38}. Thus, immobilization was characterized by measuring swimming speed (video tracking) in addition to heart rate counting. In particular, the behavioral change was measured at each exposure duration (3, 6, 12, 24, 48 hours), thus we could identify the temporal effects in detail compared to most previous studies that examined tests after 48 hours of exposure according to the OECD guidelines 202 (Fig. A1-A3 in the Supplementary Information).

To date, information on not only the temporal variations in immobilization but also the relationship between heart rate and behavioral responses to the toxic effects of nanoparticles remains limited. For example, Lovern et al. (2007) examined behavioural and physiological changes in *D. magna* using TiO₂

NPs, fullerenes and fullerene derivatives³¹. The authors found that an increased exposure level influenced the low heart rate and movement. Additionally, the effect was not statistically significant for *D. magna* exposed to TiO₂ NPs, but the temporal variation was not characterized. In our study, the heart rate increased after 48 hours of exposure compared with that after 3 hours of exposure, while it decreased in proportion to the exposure level (Fig. 2). The swimming speed at an earlier exposure duration (3 hours) was low for all materials and increased as time elapsed (Fig. 4). Therefore, it can be inferred that the exposure duration has more influences on the cardiac and behavioral effects at earlier periods, and *D. magna* are more active after a certain stabilization period.

The ROS level was additionally measured to support the outcomes in this study. The ROS levels of the target materials increased with the exposure level, but they gradually decreased from a certain level after 48 hours of exposure. The relative intensities of ROS were observed with inverted U-shaped patterns after 48 hours of exposure. This can be interpreted as the ROS levels recovered by enhanced antioxidative responses at high concentrations^{39, 40} and the overall fluorescence is enhanced by ROS production from the nanoparticles. The excessive ROS generation can cause oxidative stress⁴¹. Previous studies have also reported that oxidative stress can cause toxic effects on heart rate, swimming speed, and reproduction in *D. magna*^{42–44}. In this study, it was observed that the nanoparticles enhanced the ROS levels compared to the control group, which is also consistent with previous studies^{13, 45}.

There have been significant differences between the results on immobilization, cardiac effects and behavioral changes in previous studies. Therefore, a comprehensive test including these indices would be necessary to improve the quality of the results on acute toxicity. Furthermore, the effects of nanomaterial mixtures on aquatic toxicity need to be explored in terms of invertebrate heart physiology and swimming patterns^{46, 47}.

Methods

Experimental scheme

This study consists of three steps to examine the toxicity of nanomaterials using *D. magna*. The procedures include sample preparation (test organism, test substances), treatment (exposure), and analytical tests using instruments. One-day-old neonates that were newly born within 24 hours were prepared and certain exposure levels of nanomaterials were treated for different exposure times. After exposure duration at each concentration level, physiological effects on the neonates including the cardiac effect and behavioral changes were characterized. The test procedures for each process are described in the following sections in detail.

Daphnia magna culture

D. magna cultured in the laboratory was used in a series of experiments. The test organism was cultured in 5 L beakers in a 21 ± 1°C thermostatic incubator under a constant light cycle for 24 hours. The light

conditions were maintained in the light for 16 hours, and then the light was turned off for 8 hours (12 W, 356 mm lamp, 672 Lumen, 4200 K). To maintain the breeding condition, the *D. magna* adults were cultivated in OECD M4 medium, and daily feeding for the organisms was conducted using an algae containing a mixture of *Chlorella vulgaris* and additional nutrition (yeast, cerophyll and trout chow; YCT)⁴⁸. One-day-old neonates of *D. magna* were used for the exposure test. To check the sensitivity of the *D. magna* culture, an acute immobilization toxicity test was performed with potassium dichromate (K₂Cr₂O₇) as a reference. The sensitivity of *D. magna* culture to K₂Cr₂O₇ ranged within the limits (half-maximal effective dose, EC₅₀: 0.6–2.1 mg/L for 24 hours) suggested by OECD guideline 202. For each test concentration and control, five neonates each (< 15 hours-old) were placed in 6-well plates (VWR Tissue Culture Plates, VWR, Darmstadt, Germany) containing 10 mL of either test solution or suspension. The neonates (< 24 hours-old) obtained from the fifth generation were used in the toxicity tests to minimize variability. After incubation for 3 and 48 hours, *D. magna* was used to measure the heart rate and swimming speed.

Preparation of nanomaterials

A total of three commercially available materials including two metallic nanoparticles (TiO₂ NPs, AgNPs) and AgNO₃ were used for tests in this study. The metallic nanomaterials were purchased from manufacturers (TiO₂: Aeroxide® TiO₂ P25, Evonik Industries, Germany; AgNP: NanoXact™, NanoComposix Inc., USA) and the particle size of each nanomaterial was measured using a transmission electron microscope (TEM). Individual TiO₂ particles are normally 21 nm in size, and their aggregates are distributed hundreds of nanometers in size. Spherical AgNPs were provided at 0.02 mg/mL in a 2 mM sodium citrate solution, and the primary particle size was 31 ± 3 nm. Silver nitrate (ACS reagent, purity ≥ 99.0%; Sigma-Aldrich, St. Louis, MO, United States) was used as a source of Ag⁺. All the chemicals were used without further purification.

Determination of exposure levels

The TiO₂ NP stock solutions were made by dispersing 1 mg in 1 ml of distilled water and then sonicating for 30 min. Different suspended concentrations were prepared using ISO medium for each material. The OECD-recommended ISO medium consisted of a mixture of 294 mg/L CaCl₂·2H₂O, 123.25 mg/L MgSO₄·7H₂O, 64.75 mg/L NaHCO₃, 5.75 mg/L KCl, and 2 µg/L Na₂SeO₃. The diluted solutions of TiO₂ NPs were prepared with concentrations of 0.1, 1.0, and 10 µg/mL. The concentration levels of AgNPs and silver ions were 0.0001, 0.001, 0.01 µg/mL and 0.00001, 0.0001, 0.001 µg/mL, respectively. All diluted suspensions were immediately homogenized after vortexing. Each concentration range was determined with reference to the EC₅₀ from previous studies^{25, 35, 49–58}.

After each material was dispersed in the medium, the size distribution of each material was measured using a TEM (JEM-2100 LaB₆; JEOL, Tokyo, Japan) to identify whether the particle size was changed by medium type (distilled water vs. ISO medium) (Fig. 7). Characterization of the NP suspensions was conducted under a 200 kV accelerating voltage. An aliquot of each suspension was rinsed over a holey

carbon TEM grid (type S147-4, Plano, Wetzlar, Germany) and then dried at room temperature. More than 100 particles were taken at three magnifications due to the different particle sizes. The images were analyzed for average length (diameter) using the pixel ruler via Image J software. The primary particle size of TiO_2 was 24 ± 5 nm and the AgNP was 31 ± 3 nm (Fig. 7A & 7C). However, we found that the primary particles aggregated in the medium over 48 hours (Fig. 7B & 7D). Thus, we confirmed that *D. magna* can be exposed to primary and aggregated particles simultaneously.

Heart rate counting

The most commonly used endpoint of toxicity is to measure the death rates of *D. magna*. The immobilization of *D. magna* was observed at each concentration before counting the heart rate. LC_{50} is defined as the concentration of toxic substances that kill 50% of the test organism within a certain exposure period. Survival data were plotted and LC_{50} values were calculated using logistic 3-parameter curve fitting with Sigmaplot 13.0 software (Systat Software Inc., San Jose, CA, USA).

The heart rate was measured to evaluate the influences of exposure to each nanomaterial in triplicate. Neonates hatched within 24 hours from the fifth generation of *D. magna* adults were newly prepared on the day of the experiment. Every five neonates were exposed to each concentration level for 3 and 48 hours. The control group was also prepared for comparison with exposure groups under the same conditions without chemical treatment. After exposure, an aliquot of the methyl cellulose solution (4% V/W, Lot No. SLCC9072, Sigma-Aldrich Corp., St. Louis, MO, United States) was used to fix individual neonates on glass plate. We observed heart rate conditions for one minute using an optical microscope at 4X magnification (Model CKX41, Olympus Inc., Tokyo, Japan), and recorded them with video files. The heart rate was finally counted manually in the play condition of low speed ($\times 0.3$).

Swimming performance monitoring

As a behavioral index, the swimming performance of *D. magna* after exposure to each concentration level of nanomaterials was measured using a direct-reading instrument (Model Zebrabox, View Point Life Science Inc., Lyon, France). Individual neonates exposed to each concentration were transferred to each well of a 96-well plate separately. Each well plate batch was placed into the Zebrabox and all exposure groups were kept stable in entirely dark conditions for 30 minutes before measurement to minimize the effects of sudden environmental changes. After stabilization for 30 min, the swimming speed (distance per minute) and moving route were measured under tracking mode every minute for 80 minutes. The real-time data were analyzed using automated observation software (Zebralab-2, View Point Life Science Inc., Lyon, France).

Measurement of oxidative stress

The measurement of ROS in *D. magna* was measured using a 2, 7-dichlorofluorescein diacetate (DCFDA) cellular ROS detection assay kit (ab113851, Abcam, Berlin, Germany). After nanoparticle exposure for 3 and 48 hours, 10 for each concentration of *D. magna* were washed in a beaker with pure water and

transferred to Eppendorf tubes with 200 μ L of phosphate buffered saline (PBS). *D. magna* was homogenized by VWR® Disposable Pellet Mixers and Cordless Motor (VWR, Darmstadt, Germany). Then, the homogenates were centrifuged at 13,000 x g for 20 minutes, and the supernatant of samples was collected. Samples were kept at -80 °C until the assay was performed. Twenty microliters of each collected supernatant and 80 μ L of assay buffer were placed on a black 96-well microplate (Thermo-Scientific, Karlsruhe, Germany) and treated with 100 μ L of 10 μ M DCFDA. Then, fluorescence measurements were conducted immediately after incubation for 30 minutes in the dark using Spark® Multimode Microplate Reader (Tecan Trading AG, Männedorf, Switzerland). The excitation state was maintained at a wavelength of 485 nm, and the emission state was maintained at a wavelength of 535 nm. Each concentration was measured five times and the mean value was obtained. The total protein content of each sample was quantified by a BCA protein assay for the normalization of samples. The DCFDA levels in the *D. magna* were also visualized using a Zeiss SteREO Discovery V8 microscope with a Plan S 1.0×FWD 81 mm objective (Carl Zeiss NTS, Ltd., Jena, Germany). After that, the captured images were analyzed using ZEN imaging software (Carl Zeiss NTS Ltd., Jena, Germany).

Declarations

Acknowledgements

This work was supported by the Basic Science Research Program (Grant No. 2019R1A6A3A03033990) through the National Research Foundation (NRF) funded by the Ministry of Education, and by the Nanomaterial Technology Development Program (Grant No. 2017M3A7B6052455) funded by the Ministry of Science & ICT, and by the Technology Innovation Program (Grant No. 20011630) funded by the Ministry of Trade, Industry & Energy. We do appreciate to Dr. Marcus Koch (INM, Saarbrücken, Germany) for TEM imaging.

Author contributions

J. Park: Data Curation, Formal Analysis, Investigation, Validation, Visualization, Writing-Original Draft Preparation, Writing-Review & Editing; C.G. Park: Data Curation, Formal Analysis, Investigation, Validation, Writing-Original Draft Preparation; B. Sung: Conceptualization, Validation, Writing-Review & Editing; Y.O. Lee: Conceptualization, Validation, Writing-Review & Editing; C.S. Ryu: Conceptualization, Validation, Writing-Review & Editing; Y.J. Kim: Conceptualization, Supervision, Validation, Writing-Review & Editing; J. Park (corresponding author): Data Curation, Formal Analysis, Investigation, Validation, Writing-Original Draft Preparation, Writing-Review & Editing.

Competing interests

The authors declare there is no conflict of interest.

References

1. Aschberger, K. *et al.* Considerations on information needs for nanomaterials in consumer products. Preprint at <https://iacmcolor.org/wp-content/uploads/2014/08/JRC-Consumer-Product-Labeling-for-nano-Policy-Report-0414.pdf> (2014).
2. Boverhof, D. R. & David, R. M. Nanomaterial characterization: considerations and needs for hazard assessment and safety evaluation. *Anal. Bioanal. Chem.* **396**, 953–961 (2010).
3. Oomen, A., Bennink, M., van Engelen, J. & Sips, A. Nanomaterial in consumer products: Detection, characterisation and interpretation. Preprint at <https://rivm.openrepository.com/handle/10029/257129> (2011).
4. Hartmann, N. I. B. *et al.* Environmental fate and behaviour of nanomaterials: new knowledge on important transformation processes. The Danish Environmental Protection Agency. Environmental Project No. 1594(2014).
5. Cedervall, T., Hansson, L. A., Lard, M., Frohm, B. & Linse, S. Food chain transport of nanoparticles affects behaviour and fat metabolism in fish. *PloS One*. **7**, e32254 (2012).
6. Hou, J. *et al.* Molecular toxicity of metal oxide nanoparticles in Danio rerio. *Environ. Sci. Technol.* **52**, 7996–8004 (2018).
7. Lu, G., Yang, H., Xia, J., Zong, Y. & Liu, J. Toxicity of Cu and Cr nanoparticles to Daphnia magna. *Water Air Soil Pollut.* **228**, 18 (2017).
8. De Marchi, L. *et al.* The impacts of seawater acidification on Ruditapes philippinarum sensitivity to carbon nanoparticles. *Environ. Sci. Nano.* **4**, 1692–1704 (2017).
9. Ruppert, E. E., Barnes, R. D. & Fox, R. S. Invertebrate zoology: a functional evolutionary approach. (ed. Brooks Cole)(Cengage Learning 7th edition, 2003).
10. European Chemicals Bureau. Technical Guidance Document on Risk Assessment-Part 1. Preprint at https://echa.europa.eu/documents/10162/16960216/tgdpart1_2ed_en.pdf. (2003).
11. Tangaa, S. R., Selck, H., Winther-Nielsen, M. & Khan, F. R. Trophic transfer of metal-based nanoparticles in aquatic environments: a review and recommendations for future research focus. *Environ. Sci. Nano.* **3**, 966–981 (2016).
12. Fu, P. P., Xia, Q., Hwang, H. M., Ray, P. C. & Yu, H. Mechanisms of nanotoxicity: generation of reactive oxygen species. *J. Food Drug Anal.* **22**, 64–75 (2014).
13. Kim, T. H. *et al.* Size-dependent cellular toxicity of silver nanoparticles. *J. Biomed. Mater. Res. A.* **100**, 1033–1043 (2012).
14. Miller, M. R. Oxidative stress and the cardiovascular effects of air pollution. *Free Radical Bio. Med.* **151**, 69–87 (2020).
15. Xia, T., Li, N. & Nel, A. E. Potential health impact of nanoparticles. *Annu. Rev. Publ. Health.* **30**, 137–150 (2009).
16. Pietroiusti, A., Campagnolo, L. & Fadeel, B. Interactions of engineered nanoparticles with organs protected by internal biological barriers. *Small.* **9**, 1557–1572 (2013).

17. Pietroiusti, A. *et al.* Nanomaterial exposure, toxicity, and impact on human health. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **10**, e1513 (2018).
18. Gattoo, M. A. *et al.* Physicochemical properties of nanomaterials: implication in associated toxic manifestations. *BioMed. Res. Int.* 2014, 498420 (2014).
19. Navya, P. & Daima, H. K. Rational engineering of physicochemical properties of nanomaterials for biomedical applications with nanotoxicological perspectives. *Nano Conver.* **3**, 1 (2016).
20. Hou, J. *et al.* Toxicity and mechanisms of action of titanium dioxide nanoparticles in living organisms. *J. Environ. Sci.* **75**, 40–53 (2019).
21. Rizk, M. Z. *et al.* Toxicity of titanium dioxide nanoparticles: effect of dose and time on biochemical disturbance, oxidative stress and genotoxicity in mice. *Biomed. Pharmacother.* **90**, 466–472 (2017).
22. Nomiya, K. *et al.* Synthesis and structural characterization of silver (I), aluminium (III) and cobalt (II) complexes with 4-isopropyltropolone (hinokitiol) showing noteworthy biological activities. Action of silver (I)-oxygen bonding complexes on the antimicrobial activities. *J. Inorg. Biochem.* **98**, 46–60 (2004).
23. Sarmast, M., Salehi, H. & Khosh-Khui, M. Nano silver treatment is effective in reducing bacterial contaminations of *Araucaria excelsa* R. Br. var. *glauca* explants. *Acta Biol. Hung.* **62**, 477–484 (2011).
24. Seitz, F. *et al.* Effects of silver nanoparticle properties, media pH and dissolved organic matter on toxicity to *Daphnia magna*. *Ecotox. Environ. Safe.* **111**, 263–270 (2015).
25. Asghari, S. *et al.* Toxicity of various silver nanoparticles compared to silver ions in *Daphnia magna*. *J. Nanobiotechnol.* **10**, 14 (2012).
26. Hu, Y., Chen, X., Yang, K. & Lin, D. Distinct toxicity of silver nanoparticles and silver nitrate to *Daphnia magna* in M4 medium and surface water. *Sci. Total Environ.* **618**, 838–846 (2018).
27. Martins, J., Teles, L. O. & Vasconcelos, V. Assays with *Daphnia magna* and *Danio rerio* as alert systems in aquatic toxicology. *Environ. Int.* **33**, 414–425 (2007).
28. Bownik, A. *Daphnia* swimming behaviour as a biomarker in toxicity assessment: a review. *Sci. Total Environ.* **601**, 194–205 (2017).
29. Kunze, J., Hartmann, S., Witte, K. & Kuhnert, K. D. *Daphnia magna* as biosensor for Ag-nanoparticles in water systems: Development of a computer vision system for the detection of behavioral changes. Preprint at <http://homepages.inf.ed.ac.uk/rbf/VAIB16PAPERS/vaibkunze.pdf> (2016).
30. Lovern, S. B. & Klaper, R. *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C60) nanoparticles. *Environ. Toxicol. Chem.* **25**, 1132–1137 (2006).
31. Lovern, S. B., Strickler, J. R. & Klaper, R. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C60, and C60HxC70Hx). *Environ. Sci. Technol.* **41**, 4465–4470 (2007).
32. Simão, F. C. *et al.* Using a new high-throughput video-tracking platform to assess behavioural changes in *Daphnia magna* exposed to neuro-active drugs. *Sci. Total Environ.* **662**, 160–167 (2019).

33. Fekete-Kertész, I. *et al.* How Does Experimental Design Modify the Result of *Daphnia magna* Heartbeat Rate Test? Analyses of Factors Affecting the Sensitivity of the Test System. *Period. Polytech-Chem.* **62**, 257–264 (2018).
34. Chung, W., Song, J. M. & Lee, J. The Evaluation of titanium dioxide nanoparticle effects on cardiac and swimming performance of *Daphnia magna*. *Int. J. Appl. Environ. Sci.* **11**, 1375–1385 (2016).
35. Ribeiro, F. *et al.* Silver nanoparticles and silver nitrate induce high toxicity to *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Danio rerio*. *Sci. Total Environ.* **466**, 232–241 (2014).
36. Shen, M. H. *et al.* Exposure medium: key in identifying free Ag⁺ as the exclusive species of silver nanoparticles with acute toxicity to *Daphnia magna*. *Sci. Rep.* **5**, 9674 (2015).
37. Organization for Economic Cooperation and Development (OECD). OECD guideline for testing of chemicals – *Daphnia* sp., Acute immobilization test. Preprint at https://www.oecd-ilibrary.org/environment/test-no-202-daphnia-sp-acute-immobilisation-test_9789264069947-en (2002).
38. Mora-Zamorano, F. X., Larson, J. K. & Carvan, M. J. Neurobehavioral analysis methods for adverse outcome pathway (AOP) models and risk assessment in A Systems Biology Approach to Advancing Adverse Outcome Pathways for Risk Assessment (ed. Garcia-Reyero, N.) 149–175 (Springer, 2018).
39. Galdiero, E. *et al.* An integrated study on antimicrobial activity and ecotoxicity of quantum dots and quantum dots coated with the antimicrobial peptide indolicidin. *Int. J. Nanomed.* **11**, 4199 (2016).
40. Zhou, H. *et al.* B11 alleviates cardiac microvascular ischemia-reperfusion injury via modifying mitochondrial fission and inhibiting XO/ROS/F-actin pathways. *J. Cell. Physiol.* **234**, 5056–5069 (2019).
41. Klaper, R. *et al.* Toxicity biomarker expression in daphnids exposed to manufactured nanoparticles: changes in toxicity with functionalization. *Environ. Pollut.* **157**, 1152–1156 (2009).
42. Bownik, A. & Stępniewska, Z. Protective effects of ectoine on behavioral, physiological and biochemical parameters of *Daphnia magna* subjected to hydrogen peroxide. *Com. Biochem. Phys. C.* **170**, 38–49 (2015).
43. De Felice, B., Salgueiro-González, N., Castiglioni, S., Saino, N. & Parolini, M. Biochemical and behavioral effects induced by cocaine exposure to *Daphnia magna*. *Sci. Total Environ.* **689**, 141–148 (2019).
44. Liu, Z. *et al.* Effects of nanoplastics at predicted environmental concentration on *Daphnia pulex* after exposure through multiple generations.. *Environ. Pollut.* **256**, 113506 (2020).
45. Lu, H., Fan, W., Dong, H. & Liu, L. Dependence of the irradiation conditions and crystalline phases of TiO₂ nanoparticles on their toxicity to *Daphnia magna*. *Environ. Sci. Nano.* **4**, 406–414 (2017).
46. Baek, M. J. *et al.* Quantitative prediction of mixture toxicity of AgNO₃ and ZnO nanoparticles on *Daphnia magna*. *Sci. Technol. Adv. Mater.* **21**, 333–345 (2020).
47. Park, C. B. *et al.* Mixture toxicity of metal oxide nanoparticles and silver ions on *Daphnia magna*. *J. Nanopart. Res.* **21**, 166 (2019).

48. National Institute of Environmental Research (NIER). Operational guideline for ecotoxicity test procedures. Document No. NIER-GP2015-094. Preprint at <https://ecolibrary.me.go.kr/nier/#/search/detail/5595086> (in Korean) (2015).
49. Baumann, J., Sakka, Y., Bertrand, C., Köser, J. & Filser, J. Adaptation of the *Daphnia* sp. acute toxicity test: miniaturization and prolongation for the testing of nanomaterials. *Environ. Sci. Pollut. R.* **21**, 2201–2213 (2014).
50. Cupi, D., Hartmann, N. B. & Baun, A. Influence of pH and media composition on suspension stability of silver, zinc oxide, and titanium dioxide nanoparticles and immobilization of *Daphnia magna* under guideline testing conditions. *Ecotoxicol. Environ. Safe.* **127**, 144–152 (2016).
51. Georgantzopoulou, A. *et al.* Ag nanoparticles: size-and surface-dependent effects on model aquatic organisms and uptake evaluation with NanoSIMS. *Nanotoxicology.* **7**, 1168–1178 (2012).
52. Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H. C. & Kahru, A. Toxicity of nanosized and bulk ZnO, CuO and TiO₂ to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere.* **71**, 1308–1316 (2008).
53. Hlavkova, D., Havelkova, B., Kopel, P. & Beklova, M. Evaluation of nanosilver ecotoxicity using representatives of distinct trophic levels. *Fresenius Environ. Bull.* **28**, 745–749 (2019).
54. Hurel, C. *et al.* Functionalized and grafted TiO₂, CeO₂, and SiO₂ nanoparticles—ecotoxicity on *Daphnia magna* and relevance of ecofriendly polymeric networks. *Environ. Sci. Pollut. R.* **25**, 21216–21223 (2018).
55. Jemec, A. *et al.* An interlaboratory comparison of nanosilver characterisation and hazard identification: Harmonising techniques for high quality data. *Environ. Int.* **87**, 20–32 (2016).
56. Kim, J., Kim, S. & Lee, S. Differentiation of the toxicities of silver nanoparticles and silver ions to the Japanese medaka (*Oryzias latipes*) and the cladoceran *Daphnia magna*. *Nanotoxicology.* **5**, 208–214 (2011).
57. Marcone, G. P., Oliveira, ã. C., Almeida, G., Umbuzeiro, G. A. & Jardim, W. F. Ecotoxicity of TiO₂ to *Daphnia similis* under irradiation. *J. Hazard. Mater.* **211**, 436–442 (2012).
58. Picado, A. *et al.* A multi-integrated approach on toxicity effects of engineered TiO₂ nanoparticles. *Front. Env. Sci. Eng.* **9**, 793–803 (2015).

Figures

Figure 1

Viability of *D. magna* as a function of TiO₂ and Ag nanoparticles (NPs) and Ag⁺.

Figure 2

D. magna heart rate (BPM, beats per minute) changes after 3 and 48 hours of exposure to TiO₂, AgNPs and Ag⁺ at varying concentrations. Data are presented as the mean±standard deviation (n=3).

Substance	Exposure level (µg/ml)		
Control Group	0		
TiO ₂	0.1	1.0	10
Ag NP	0.0001	0.001	0.01
Ag ion	0.00001	0.0001	0.001

Figure 3

Representative images of the *D. magna* swimming paths for the initial 10 minutes as a function of the concentration of TiO₂, AgNPs and Ag⁺.

Figure 4

Plots of *D. magna* swimming speed as a function of the concentration of TiO_2 , AgNPs and Ag^+ and exposure time (up to 48 hours).

Figure 5

Representative optical microscopic images of *D. magna* exposed to Ag⁺, AgNPs, and TiO₂ NPs for 3 hours. Each image set represents bright field, fluorescence, and merged images, from left to right. Scale bars, 200 µm.

Figure 6

Effects of TiO_2 and Ag NPs and Ag^+ on reactive oxygen species (ROS) levels in *D. magna* after 3 and 48 hours of exposure. Data are presented as the mean $\pm$ standard deviation ($n=3$).

Figure 7

TEM images of the nanomaterials; (a) TiO₂ NPs in distilled water. (b) TiO₂ NPs in ISO medium. (c) AgNPs in distilled water. (d) AgNPs in ISO medium. Scale bars, 200 nm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Sci.Rep.SupplementaryInformationRev..docx](#)