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Ecotoxicity of nano-metal oxides: A case study on daphnia magna

Monia Renzi [1] · Andrea Blašković[1]

Abstract

In Europe REACH framework directive imposes data acquisition concerning toxicity on acquatic species before the commercialization of chemicals to assess environmental risks. According to official methods, exposure tests are performed under in vitro and standardized conditions: OECD's guideline rules external variables such as water type, feeding conditions, and exposure time. As consequence, such obtained results could be different from effects observed in natural environments. This study collects effects within 24–96 h of exposure to nano metal-oxides (ZnO, TiO_2) on *D. magna* obtained by the exposure under standard OECD conditions comparing them with results obtained by the exposure under more similar conditions to natural environment (i.e. mixture, feeding). High doses exposure determines gas-bubble disease. Animals exposed to LC10 actively ingest nanoparticles under both fasting and feeding conditions. Furthermore, body burial by a coat of nanoparticles thicker in mixtures than in single dispersions was recorded. Furthermore, results show that: (i) effects increase over time; (ii) n-ZnO results less effective than n-TiO_2 in both single dispersion, and mixture; (iii) the presence of surfactant increases toxicity of nanoparticles; (iv) immobilization is a more sensitive endpoint than mortality; (v) feeding increases test sensitiveness improving differences among treated and controls till 96 h and allowing longer exposure times than standard OECD test. As general remark, this study provides evidence that in vitro ecotoxicological results obtained under standardized OECD conditions could be significant different to animals' responses under natural (feeding and mixtures) exposure conditions.

Keywords Metal-oxide nanoparticles · REACH · Toxicity tests · Cladocerans · OECD

Introduction

Nanoparticles in aquatic environment: a brief overview

The hetherogeneous group of chemicals originated from different processes and materials are classifiable under the umbrella term "nanoparticles" (Moore 2006) if sized within 1–100 nm which affect their functionality (Pettitt and Lead 2013). Industrial application involving nanoparticles (NPs) is growing worldwide (Maynard 2006) both for NPs of natural origin (i.e. humic and fulvic acids, fullerenes, organic acids, and metals) and for artificial NPs such as TiO_2, ZnO (Nowack and Bucheli 2007; Kumar et al. 2014). Pharmaceutical and personal care products, plastic, rubber, paints, glass and many other products of common use are based on NPs (WWC 2013; Hossain et al. 2014). Among emission sources of NPs, urban municipal wastewater treatment plants represent significant sources for aquatic ecosystems (Liu et al. 2013). In fact, more than indirect sources, intentional releases of nano-oxides for the sorptive removal of organic contaminants from wastewater (Jing et al. 2013) purification purposes are increasing. Ecotoxicity increases with the reduction of NPs size (Sun et al. 2009) and the scientific interest towards their effects on biota quickly increses (Ducrotoy and Mazik 2011; Wang et al. 2014). Recent studies highlight concrete eco-toxicological effects and exposure risks (Gottschalk et al. 2009; Mukherjee and Acharya 2018) for aquatic ecosystems. In spite of the increasing interest, recent meta-data analysis highlighted low standardization of in vitro tests (Renzi Gurranti 2015) and the largest amount of studies performed on simple NPs matrices.

In the European Union, data on ecotoxicological effects of nanoparticles were recently improve under the application of

✉ Monia Renzi
monia.renzi@bsrc.it

[1] Bioscience Research Center, Via Aurelia Vecchia, 32, 58015 Orbetello (GR), Italy

REACH framework Directive (Pettitt and Lead 2013). According to REACH, ecotoxicity for the aquatic environment should be tested before the commercialization of chemicals and/or mixtures. Tests should be performed in vitro on species considered representatives of the aquatic environments including cladocerans according the Organization for Economic Cooperation and Development (OECD) testing guidelines. Results are associated to the commercialized product as descriptive of possible risks for the aquatic environment (Hund-Rinke et al. 2018). OECD tests are performed under standardized conditions on commercial formulations; any information is obtained on effects of "natural" mixtures among nutrients, NPs, and surfactants that occurr in MWWTP.

Why to explore interactive responses in the evaluation of the ecotoxicological effect?

Toxic effects coming from the exposure to complex mixtures differ compared to the exposure to single substances although compounds are present at low concentrations (Schwarzenbach et al. 2006). Municipal wastewater treatment plants (MWWTP) are hot-spot sources of a wide range of pollutants from human activities (Renzi et al. 2009) for the aquatic environments that are spread as complex mixture. Nutrients (Renzi et al. 2009), surfactants (Renzi et al. 2012), and NPs (i.e. 17.1 µg/kg; Gottschalk et al. 2009) are significant components of mixtures released by MWWTP. In this case, NPs toxicity could be affected both by the nutrient-induced microalgal growth and by synergic/antagonistic interactive effects due to surfactants. Recent studies provide evidences that NPs associated to cationic surfactants produced metal-oxides nanoparticles/surfactants mixtues acting as a superior sorbent (Renzi and Gurranti 2015). The direct and indirect presence of metal-oxides NPs and surfactants in effluents from MWWTP suggests to better explore their ecotoxicity to better assess effects on aquatic environments.

This study aims to fill some important knowledge gaps on NPs ecotoxicity. It compares ecotoxicological responses of *D. magna* exposed to metal-oxides nanoparticles (commercial form of n-ZnO or n-TiO$_2$) under in vitro OECD standardized conditions to results obtained by the exposure to complex mixture (NPs + surfactant). Effects obtained under fasting conditions (OECD guidelines) are compared to results obtained during the exposure of animals under feeding conditions that are assumed as natural.

Materials and methods

Preliminary screenings

Pre-tests were performed to determine for each toxicant the dose that allows survival of a fraction of the tested population till the end of the selected exposure time (96 h). On the basis of results reported by literature (Hund-Rinke et al. 2018), during pre-tests, concentrated nanoparticles dispersions were made and, starting from 425 mg/L for n-TiO$_2$ and 500 mg/L for n-ZnO, several geometric dilutions were tested to define optimal concentrations to perform our experiments testing nanoparticles under the OECD 202:2004 guideline. Dispersions were made by suspension of tested nanoparticles in UNI EN ISO 6341:2012 standard freshwater. Suspensions were homogenized by vortexes for 20 s at 2000 rpm (Ozkan et al. 2015; Ates et al. 2013). During pre-tests a statistically significant number ($n = 30$ from experimental replicates = 3) of animals obtained by ephippia (MicroBioTest Inc., Daphtoxkit FTM magna 1996) were exposed to n-TiO$_2$ (425 mg/L) and n-ZnO (500 mg/L) to test morphological effects induced by the exposure to high concentrations.

Experimental design

On the basis of the results obtained by pre-tests, doses associated to LC10 after 24 h of exposure were fixed for experiments. We decided to test LC10 allowing exposure temporal lenght of 96 h. Selected doses were respectively 1.12 and 113.18 mg/L for n-ZnO and n-TiO$_2$. Our choices are consistent with the literature during an 8:16 dark/light exposure cycle tested under fasting conditions (Khoshnood et al. 2016). Surfactant (Triton X-100, CAS n. 9002-93-1) was tested at 0.001% v/v as reported by the literature (Frydkjær et al. 2017). The experimental design was divided into two different batches as summarized in Fig. 1. During the first batch, animals were exposed to single dispersions/solution and compared to controls to test ecotoxicological effects observed by the exposure to low doses (LC10) of n-ZnO, n-TiO$_2$, and Triton X-100. Disperions/solutions were made by suspension of tested toxicants in
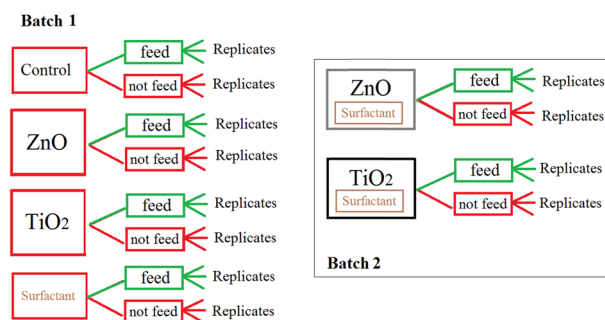

Fig. 1 Logic model adopted to perform exposure experiments. Two experimental batches are reported in figure: Batch 1—Animal exposure to single dispersions (ZnO; TiO$_2$) and solution (Triton X-100) compared to control (ISO 6341 freshwater). Red lines represent OECD 202 standard exposure protocol (fasting conditions) otherwise, green lines represent feeding animals. Batch 2—Exposure to mixtures during both fasting and feeding conditions

ISO 6341 freshwater and vortexes as previously described for pre-tests. Dispersions of both single metal-oxides NPs and mixtures were charcaterized by µFT-IR technique by ATR and microscopic imaging to evaluate the occurrence of nanoparticles cluster formation at the micrometric scale level. Furthermore, analyses were performed, also, after the addition of feeding to the testing materials to evaluate the effect induced by the presence of algae on nano-aggregates. Tests were performed following the OECD 202:2004 guideline. Animals were exposed under fasting conditions, selecting immobilization as endpoint and a contact time 24–48 h. Furthermore, to evaluate possible bias among results obtained under OECD standard conditions (considerd similar to data collected according to REACH requirements for in vitro tests on Cladocerans) and more "natural" responses, we also performed tests by the introduction of some variables of specific interest. Contextually to standard conditions required by OECD, animals were also exposed under feeding conditions, mortality was added to immobilization as selected endpoint, and contact time was extended from 24–48 h to 96 h as suggested by the literature for tests performed on particulate toxicant (Baumann et al. 2014) performing observations daily starting from T24 after the initial exposure. In the second batch, the effect of a mixture between NPs and surfactant (Triton X-100) on observed toxicity was tested and the same experimental model previously described was reproduced testing mixtures in spite that single substances (Fig. 1).

Materials and instruments

The dormant stage of *D. magna*, ephippia, were purchased by Ecotox Italia (MicroBioTest Inc., Daphtoxkit FTM magna 1996) stored in darkness, at 5 °C till analyses and hatched in ISO 6341 freshwater at 18–22 °C and 6000 lux for 72–84 h. According to the fact that neonates should not be older than 24 h at the start of the toxicity test, collection of organisms was standardized at 90 h after the start of incubation. Caelo or Sigma-Aldrich purchased all reagents. As regards as nanoparticles, analyses performed on TiO_2 (CAS n. 13463-67-7) reported particle size <21 nm and surface area of 35–65 m^2/g; while ZnO (CAS n. 1314-13-2) reported particle size <100 nm and surface area of 15–25 m^2/g. We tested commercial products as well as they were used in cosmetics for antiUV activities (TiO_2) and antimicrobial activitiy (ZnO) as ingredient as commercialized. Commercial products are more representative of the environmental behaviour of nanoparticles as well as specific functionalization processes were performed before packaging (Nowack and Bucheli 2007). Qualitative analyses of observed anomalies were performed by stereomicroscopy (Nikon, SMZ-800 N). Images were taken without removing normal and healthy animals from the plate to avoid disturb and post processed by the Nikon's software for the imaging analysis (Nikon ACT-1).

Pre-feeding

Early stages were fed by Spirulina spp. (SP200114) growth in ISO 6341 freshwater; all animals used for exposure tests were fed before starting experiments to allow a longer exposure time (96 h). After a contact time of almost 2 h (density of about 10^4 cells), animals exposed under fasting conditions were incubated in ISO 6341 freshwater for 1 h as performed by Jemec et al. (2016) and successively were exposed to toxicants.

Test conditions and experimental controls

Animals not older than 24 h were exposed in 10 mL of dispersion/solution in triplicates of tested solutions and experimental controls. Temperature was constantly monitored during experiments. Tests were performed at 18 °C (17–19 °C max. range allowed) under dark conditions to evaluate effects under low metabolic levels. Furthermore, concerning TiO_2 nanoparticles, light exposure resulted effective to change toxicity to aquatic biota due to their photocatalytic properties (Clemente et al. 2013). Even if experiments by Clemente et al. (2013) were performed on fish species, our experiments were performed by dark to avoid photocatalytic interactions. In fact, recent literature reported reactivity in the presence of illumination (Hund-Rinke 2018). Darkness is also allowed by OECD guidelines, which it does not standardize photoperiod. Tested platforms were not aerated during exposure to avoid disturbance and to reduce evaporation rates and external airborne contaminations the test platforms were covered with a plastic cap. Animals tested under feeding conditions, were fed with Spirulina spp. commercial powder once a day after counting. Dissolved oxygen and pH were measured at the end of the experiments to check the standard requirements in the life range of tested animals. At each time, animals were counted to evaluate mortality, immobilization. Also, anomalous behaviour/appearance was recorded. Data are expressed as percentages of survived animals (100-mortality%) and as percentages of active animals (100-immobilized%) at each exposure time. Three experimental plates (3 independent replicates) were performed. Animals were considered immobilized if they lose capability to perform vertical swim after 15 s of observation and after a gentile agitation of the test platform.

Quality control & Quality assurance

BsRC (certified laboratory ISO 9001:2015 and ACCREDIA Lab. N. 1715) ensure data quality by the application of a

severe control procedure under guidelines of the UNI EN ISO 17025 to ensure quality of data. Inter-calibration exercises on ecotoxicological tests were passed on annual basis by our laboratory testing *D. magna* responses. Our performances on *D. magna* resulted optimal. Animals were exposed to standard ISO 6341 freshwater medium. Initial water parameters (pH, DO) were monitored and controlled to be included within the range of acceptability for the tested species (see OECD 202:2004 reference values for testing water; see Supplementary material). Tests were considered valid if oxygen levels in tested and control samples at the end of the exposure test were >60% of the air saturation value. Both negative and positive controls were performed during experiments to test experimental conditions and animal standard responses. Tests were considered valid if mortality in negative controls does not exceeded 10% after 24 h of exposure without feeding following the acceptability criteria reported by OECD guideline (OECD 202:2004). Standard response of *D. magna* to toxicant (positive control) was tested as LC50 after 24 h of exposure with potassium dichromate ($K_2Cr_2O_7$). The LC50 value for reference chemical was calculated from the concentration-effect curves by the application of the lognormal model using REGTOX software (available online at: http://eric.vindimian.9online.fr/.). Tested animals resulted within the acceptable range of 0.6–2.1 mg/L. Significance ($p < 0.01$) of observed differences among mean values was tested by T-test while differences among variances were explored by F-test (Prism® 4.0)

Results

Results are reported as fraction of animal survived (%) and fraction of active animal (%) respectively after 24 h (T24), 48 h (T48), 72 h (T72), and 96 h (T96) of exposure. Results are summarized according to fasting and feeding conditions and compared to negative controls. The exposure of *D. magna* at high concentrations during pre-tests produces significant morphological effects as reported in Fig. 2. In Fig. 3, the effects on animals exposed to tested

concentrations closed to LC10 levels are reported. Results related to the measurement of both living and active animals under fasting and feeding conditions are reported in Fig. 4a–d. In particular, in Fig. 4a and Fig. 4b, per exposure time (T0-T96), data concerning survived animals (%) respectively under fasting and feeding conditions are reported. On the contrary, in Fig. 4c, d active animals (%) are reported respectively under fasting and feeding conditions. A comparative analysis between effects measured under fasting and feeding conditions is reported in Table 1.

Morphological damages

Nanoparticles immobilize animals by absorption to their body surface and to their appendices. Large quantities of nanoparticles are ingested up by exposed animals to completely fill their gastrointestinal tract. Furthermore, high exposure doses stimulate the development of large gaseous bubbles inside the animals. Described phenomena determine the rapid death of exposed *D. magna* occurred after only 24 h of exposure to high concentrations (Fig. 2).

Animals actively ingest nanoparticles during both fast and feeding conditions. Algal accumulation inside the digestive system under feeding conditions is well visible (greenish yellow spherules). Intakes of nanoparticles are well visible as black colouring of the digestive tract. Concerning the second experimental batch, as interactive effect of nanoparticles and surfactant, a greater number of nanoparticles adsorbed on the surface of *D. magna*'s carapace is recorded. Coverage by external coat resulted ticker in exposure to mixtures than to single dispersions (Fig. 3).

Negative controls (ISO 6341 freshwater)

After 24 h of exposure (T24), mortality resulted closed to zero (100% survival, Fig. 4a) in negative controls. OECD guideline does not define a range of acceptability for this endpoint. After the same time of exposure, the average percentage of immobilized animals (Fig. 4c) is higher if compared to died ones ($9.6 \pm 5.6\%$ versus 0% respectively), nevertheless, results are within the range of acceptability of

Fig. 2 Effects on exposed animals with extreme concentrations. Effects observed at high concentrations of TiO_2 (425 mg/L) during 24 h exposure: **a** gas bubble formations inside the animals; **b** fast mortality due to complete immobilization of the animal
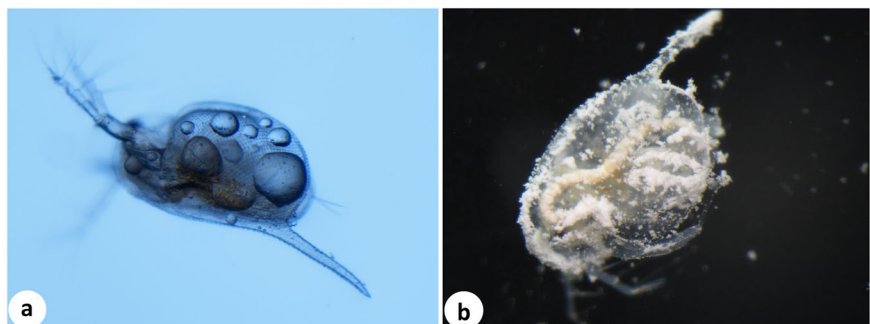
Fig. 3 Effects on exposed
animals. **a** fasting controls; **b** fed
controls (algal accumulation is
visible inside the digestive
system; **c** fasting exposed
animals to nanoparticles. Black
digestive system is due to
nanoparticles intakes; **d**
interactive effect of
nanoparticles and surfactant
(Triton X-100) on the carapace
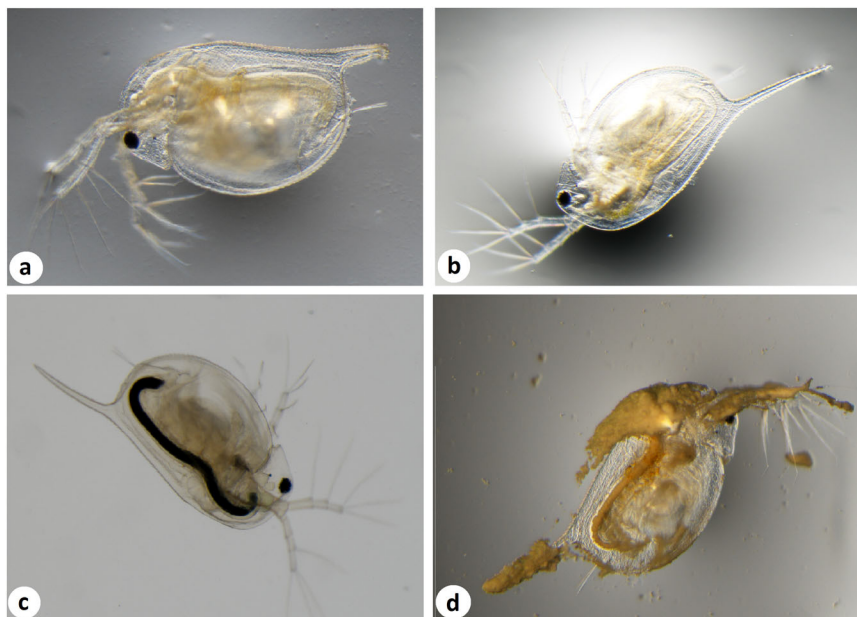of *D. magna* and on the
digestive system

Fig. 4 Effects observed during
the exposure under fasting and
feeding conditions. Results
represented **a-d** summarize
average (SD) living animals %
(calculated as 100-died %) after
the exposure under fasting (**a**)
and feeding (**b**) conditions and
active animals % (calculated as
100-immobilized %) under the
same fasting (**c**) and feeding (**d**)
conditions. Green lines represent
ISO 6341 freshwater (negative
controls). Results are
represented according to the
exposure time; data are collected
daily (each 24 h of exposure)
from T0 to T96. Geometric
figures represent tested
disperions and solution (full
black represent single solutions
while empty ones represent
mixtures). Standard deviations
are also reported. Oxygen levels
in tested and control samples at
the end of the exposure test were
respectively within 95–98% and
85–93%
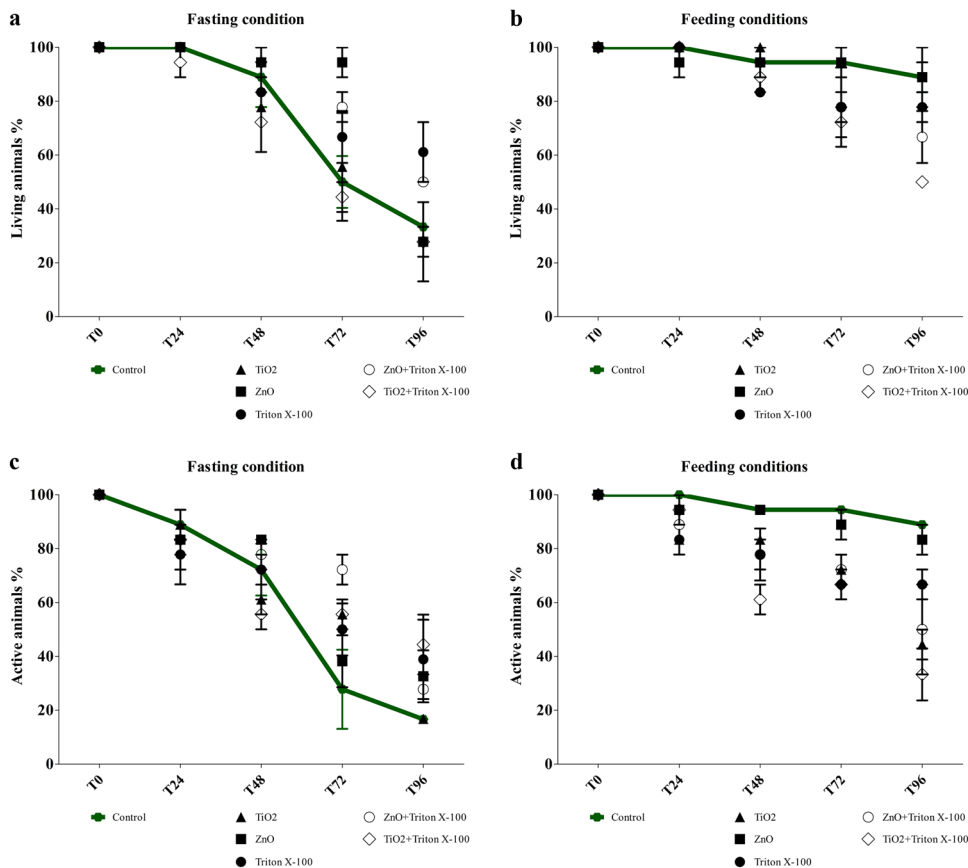
the test reported by OECD 202 guideline. After 24 h,
mortality quickly increased over time growing from $11.0 \pm 19.2\%$ (T48) up to $66.7 \pm 9.6\%$ (T96) under fasting condi-
tions. A similar trend over time was observed considering
immobilized animals as endpoint (Fig. 4c). In fact, per-
centages of immobilized animals under fasting conditions
quickly increased from $27.8 \pm 16.7\%$ (T48) to $83.3 \pm 0.0\%$
(T96). On the contrary, animals exposed under feeding

Table 1 Comparisons between effects measured under fasting and feeding conditions

Endpoint (%)	Time	Single dispersion/solution			Mixtures	
		TiO$_2$	ZnO	Titron X-100	TiO$_2$ Triton X-100	ZnO Triton X-100
Survival	T24	0	−5.6	0	5.6	0
	T48	22.2	0	0	16.7	0
	T72	22.2	0	11.1	27.8	0
	T96	27.8	61.1	16.7	22.2	16.7
Active animals	T24	−5.6	11.1	5.5	11.1	5.6
	T48	22.2	11.1	5.6	5.5	0
	T72	16.6	50.7	16.7	11.1	0
	T96	27.6	50.7	27.8	−11.1	22.2

Results are expressed as difference between the average effects recorded under fasting and feeding conditions. Positive values mean the major average effect (%) was recorded under fasting conditions, while negative values mean the major average effect was recorded under feeding conditions. Results are grouped according the exposure batch and the exposure time

conditions showed constant and low percentages of mortality over time ($p < 0.01$). Dead animals were recorded starting from T48 ($5.6 \pm 9.6\%$) and reached $11.1 \pm 9.6\%$ (T96) at the end of the experiment (Fig. 4b). Analogous results ($p < 0.01$) were recorded considered immobilization as endpoint (Fig. 4d).

Toxicity of tested chemicals under fasting conditions

As represented in Fig. 4a, mortality of single dispersions (ZnO; TiO$_2$) and solution (Triton X-100) resulted 0% at T24. Mixtures showed a different behaviour evidencing the absence of mortality and $5.6 \pm 9.6\%$ respectively in ZnO and TiO$_2$ with the addition of Triton X-100 (T24), standard deviations are very low in all tested dispersion/solution. Starting from T48, mortality increased significantly over time following the same trend observed for negative controls. An exception was recorded for ZnO showed low mortalities ($5.6 \pm 0.0\%$) till T72 and higher mortalities than controls ($72.2 \pm 9.6\%$) in T96. At T48, TiO$_2$ shows mean mortality three times higher than control and recorded mortalities are scaled as reported: TiO$_2$ + Triton X-100 ($27.8 \pm 19.2\%$) > TiO$_2$ ($22.2 \pm 28.9\%$) > Triton X-100 ($16.7 \pm 0.0\%$) > controls ($11.1 \pm 19.2\%$) > ZnO; ZnO + Triton X-100 ($5.6 \pm 9.6\%$). It is to notice that almost all tested single dispersion/solution and mixtures showed mortalities higher than controls till T48 but, after that, controls showed higher mortality rates, compared to tested chemicals and mixtures with the only exception (+5.6%) of TiO$_2$ + Triton X-100 (T72) and ZnO; TiO$_2$ + Triton X-100 (T96).

As regards as immobilization (Fig. 4c), effects are always higher than mortality at any time. In particular, not immobilized animals are closed to 80% (77.8–88.9%) in T24 with few differences among tested chemicals; active fraction of animals reduced over time and quickly decreased. Significant differences are reported among controls and tested dispersion/solution and mixtures. In particular, T24 all tested dispersion/solution and mixtures recorded higher immobilized percentages than controls. On the contrary, T72 and T96 showed that animals are less active in controls than in tested dispersion/solution and mixtures.

Effects observed under feeding conditions

Feeding conditions showed important different behaviour compared to animals exposed under fasting conditions. In Table 1, a comparison between effects measured under fasting and feeding conditions is reported. As regards as mortality (Fig. 4b), under feeding conditions control showes higher stability and allow to perform observation till T96. ZnO showed higher mortality rates than TiO$_2$ after 48 h but this trend clearly inverted after 72–96 h of exposure. Immobilization showed the same trend observed for mortality (Fig. 4d) evidencing higher sensibility. In both cases trends over time are much more clear and higher toxicity of mixture is well evident compared to single dispersion/solution.

Discussion

Gas-bubble formation

At high doses tested during the first phase of our experiments, exposed animals died quickly (24 h). Nanoparticle toxicity is associated to significant changes of chemical conditions of water media as documented by the formation of gas bubbles inside the animal's body (see Supplementary materials for a specific discussion on this aspect).

Exposure doses closed to LC10 tested during the experiments do not provided evidences of gas-bubble deseases and recorded O$_2$, pH in water of tested media resulted within the vital range reported for these animals at the end of the experiment (T96). This fact let us to suppose different toxic mechanisms following nanoparticle exposure rather than oxygen-diseases such as body burial, intestinal damages following ingestion, direct toxicity. Weitkamp and Katz (1980) reported that even if gas bubbles appears in certain conditions of gas oversaturation together with the increasing of temperature, bubbles contains mainly nitrogen, not oxygen and this fact could explain normal levels of oxygen recorded in medium at the end of the experiments.

Body burial

In this study, we observed that exposed animals to nano metal-oxides are immobilized subsequent to body burial by a thick coating coverage. Chen et al. (2012) reported that the exposure to 50 NTU of suspended solids decreased animals' motility strength to 70% after 10 h, while 100 NTU reduced motility strength 3 h later supporting that mechanical impacts are effective to induce immobilization. Tao et al. (2011) observed in *D. magna* a body burial condition increasing with the NPs concentration. Our study shows that NPs- surfactants mixtures better induce coatings (at LC10 exposure) by clusters formation on animals' external appendices. NPs resulted not so effective in net production when animals were exposed without Triton X-100. The body burial observed produces severe effects on survival rates and on animals' mobility under any condition tested. Animals try to escape the net by moving continuously; this fact probably determined higher energy consumption by trapped animals that induced significantly higher effects under fasting conditions. In this study, tested n-TiO2 was smaller than n-ZnO favouring the formation of a thicker coating on exposed animals compared to n-ZnO. Some key factors such as surface/volume ratio, aggregation in water (Ducrotoy and Mazik 2011), uptakes, and interactions with biota (Nel et al. 2006) are case-specific and required future studies. Furthermore, significant transformations of structure, shape, and size occur in aquatic environments from aggregation, solubilisation or adsorption (Handy et al. 2008) determining different toxicity on biota.

Direct toxicity

Cladocerans are important members of freshwater aquatic ecosystems by an ecological point of view and toxicity on this species could produce significant ecological effects. Recent studies define ecotoxicity of nanoparticles on *D. magna* (Handy et al. 2008). Furthermore, Khoshnood et al. (2016), reported LC10, LC50, LC90, safety factors, and safe application rate (Jaafarzadeh et al. 2013) for n-ZnO and n-TiO2 evidencing that tested nanoparticles may have acute dose-dependent ecotoxicological effects. After the exposure to metal-oxide nanoparticle LC10 of n-TiO2 (24 h) was about 1/100 less than n-ZnO (Khoshnood et al. 2016). As regard as letal concentrations 50% (LC50) for *D. magna* following the exposure to nanoparticles metal-oxides, a wide range of values is reported by the literature. Concerning n-ZnO, recent researches reported a LC50 (T48) of 3.20 mg/L (Heinlaan et al. 2011); Liu et al. (2013) observed a LC50 twice (6.32 mg/L); while others recorded LC50 (T48) lower than previously cited researches and less variable values: 1.51 mg/L (Zhu et al. 2010); 1.00 mg/L (Wiench et al. 2009); and 1.10 mg/L (Lopes et al. 2004).

Concerning n-TiO2, LC50 (T48) values reported by the literature are significantly higher than those recorded for n-ZnO: Zhu et al. (2006) recorded for n-TiO2 a LC50 value of 143.39 mg/L. Our study showed that mortality rates observed for doses closed to LC10 (T24) are consistent with the literature (Zhu et al. 2010; Jaafarzadeh et al. 2013; Khoshnood et al. 2016). This study, performing longer exposure tests than previous literature, allowing evaluate also cronical effects over time following the exposure under LC10 doses.

The toxicity of metal-oxide nanoparticles could be due to the release of metal ions in water (Blinova et al. 2010). It was reported by the literature that toxicity of n-ZnO could be associated to Zn^{2+} release in water and that ion release is strictly associated to particle size, reaching maximum levels for 4–7 nm particles diameters (Mudunkotuwa et al. 2012). Literature, also, provided evidences that some water chemical parameters could affect n-ZnO toxicity. In particular, increasing pH and DOM reduced the concentration of free Zn^{2+} released from n-ZnO, and thus lowered the toxicity of n-ZnO. Furthermore, Ca^{2+} and Mg^{2+} dramatically reduced the toxicity of Zn^{2+}. Effects recorded in this study are associated to standard DOM, Mg^{2+}, Ca^{2+}, and pH levels as well as tests were performed using ISO 6341 standard freshwater. Feeding could have affected these key parameters (DOM and pH in particular) following the addition of an external organic source (DOM) and due to changes induced by photosynthesis performed by algal activity on water chemical features. Nevertheless, algal supplies provided during our experiments were standardized to allow the complete consumption of the provided food within few hours from the addition, so we considered negligible this possible contribute.

Results recorded after T48 of exposure showed a higher toxicity in tested dispersion/solution, and mixtures than in negative controls with the exception of n-ZnO. Literature reported that differences among particle size of tested nanoparticle as well as other inconsistent test conditions (such as pH, photoperiod, and dissolved oxygen; Wang et al. 2014), could affect observed toxicity. In this study, inconsistent conditions were fixed and standardized so, the higher toxicity of n-TiO2 than n-ZnO recorded differently than expected could be due to the difference between the average particle-size dimensions of nano metal-oxides tested in this study (tested n-TiO2 was smaller than n-ZnO).

Furthermore, on average, the presence of surfactant increased toxicity of tested dispersions starting from T24. Higher toxicity recorded at T48-96 could be due to the presence of the surfactant but results over time are difficult to be interpreter under fasting conditions due to the low gap between effects recorded in negative controls and tested samples. Compared to animals exposed to tested dispersions/solutions, a higher mortality rate and a lower animal

activity were recorded frequently in negative controls under fasting conditions starting from T48. This could be explained by the high stress occurring in the case of absence of ingestion compared to the ingestion of nanoparticles or mixtures; nevertheless, further studies are needed to better understand this observed result also associating biomarker responses. Under standard OECD conditions (T24, immobilization, fasting), tested single solutions of nano metal-oxides resulted widely under EC20 but tested concentrations quickly produced EC20 effects over times both under fasting and feeding conditions.

Avoidance behaviour

Animals exposed to toxicants showed many induced behavioural and physiological responses. Ren et al. (2009), provided evidences that the exposure to chemicals induced behavioral responses that are affected in general by exposure concentration, rather than toxic characteristics of the chemicals. Hops resulted important behaviors for escaping danger (Seely and Lutnesky 1998) and it is reported as key responding to toxic environments in sublethal concentrations (Lopes et al. 2004). On the contrary, Ren et al. (2009) suggested that behavioural changes could be due to physiological damages to visual or chemoreception apparatous even if these effects were reported by the literature on vertebrates (Mandrillon and Saglio 2007). As reported in this study, avoidance seemed to be significant higher in exposed animals rather than controls under fasting conditions during which animals showed increased velocity and more frequent hoping behaviour. Avoidance is an adaptive behaviour to reduce exposure to harmful chemicals or substances improving animal fitness and survival and well documented by the literature (Eriksson Wiklund et al. 2006; Ren et al. 2009). Ren et al. (2009) suggested from the observation that avoidance behaviors of *Daphnia magna* to exposure to chemicals with different toxic characteristics could be similar, while their specific response could be different. Behavioural changes could be associated also to feeding and biological cycles. Reynaldi et al. (2006) provided evidences that a reduction of feeding activities of exposed animals with a consequent reduction of the body size and a delay of the sexual maturation of exposed animals. Untersteiner et al. (2003), reported a decrease of the average swimming velocity testing *D. magna* at 30 ppb of Cu. Velocity reduction was recorded later in lower exposure doses experiments (14 h after the exposure at 10 ppb of Cu). It was reported by the literature that the exposure to sublethal n-TiO$_2$ concentrations induced behavioral and physiological responses in *D. magna* that could lead to significant changes in hopping frequency, feeding appendage and postabdominal curling movement, and heart rate (Lovern et al. 2007) changing the energy consumption during the exposure.

Toxicity of mixture are significantly different by toxicity observed after the exposure to single nanoparticle dispersion as also recorded by the literature as well as they could act as a carrier of co-existing contaminants (Baun et al. 2008). As observed by this study, the presence of surfactants, a frequent occurrence in effluents from MWWTPs and aquatic human-stressed environments (Renzi et al. 2009; Renzi et al. 2012), could significantly affect toxicity on this species. Furthermore, feeding condition, another natural occurrence in freshwater environments, shows significant impacts on recorded toxicity. Results proposed by this study and also reported by the literature suggest serious consideration of the consequences before random utilization of metal-oxides nanoparticles also at doses that are considered safe under in vitro experiments (Mukherjee and Acharya 2018).

Fasting and feeding effects

Nanoparticles are ingested with large efficiencies both under fasting and feeding conditions during the whole exposure tests. Ingestion of nanoparticles occured during this study regularly and nanoparticles were observed within the intestinal tract of tested animals under both fasting and feeding conditions. The presence of surfactant improves levels of nanoparticles adsorbed on the animal's carapace. Large quantities of nanoparticles are ingested up to completely fill the gastrointestinal tract of the animals. This study does not allow us to quantify differences among ingestion rates due to the presence of surfactants. The ingestion of nanoparticles was previously recorded by the literature and it was associated to a significant mortality rate due to the block of the intestinal tract associated to the formation of agglomerates (Wiench et al. 2009).

D. magna is planktonic filter feeders (Ebert 2005) and, as a predator of algae, it could take up nanoparticles through the filter-feed system (Filella et al. 2008), and ingests them (Zhu et al. 2009). It has been reported that feeding appendage of *D. magna* easily filters particles within 0.1–35 µm in size with special efficiency on particle diameters between 0.1–1µm (Gophen and Geller 1984). Particles larger than the average mesh size are retained very efficiently by daphnid (Gophen and Geller 1984; Demott 1982; Brendelerger 1991), and the particles are sent to the intestine (Zhu et al. 2010; Kukka et al. 2010) where they aggregate and block the intestine for a long period until death (Zhu et al. 2009). The larger size particles were taken up by *Daphnia spp*. quickly at the beginning of exposure (Tao et al. 2011). As regards as particle size, and uptake of particles >100 nm reached their maximums more quickly than those <100 nm under environmental conditions comparable to those standardized in this study (i.e. low hardness, higher pH, lower temperature; Tao et al. 2011). Smaller particles less than 100 nm exhibit less protein absorption than those greater

than 100 nm (Fang et al. 2006), therefore smaller particles can be taken up and not only stored in the lipid of the body (Tao et al. 2011) but also stored in protein. This is probably the reason for the higher mortality of n-TiO$_2$ than n-ZnO observed and associated to the smallest average dimensions of n-TiO$_2$ compared to n-ZnO even if the latter is reported by the literature to be much more toxic than n-TiO$_2$.

Ingestion of nanoparticles could represent a significant effect in this study as well as exposed animals resulted completely affected by the ingestion of nanoparticles under both fasting and feeding conditions. In our study, under feeding conditions, immobilized percentages are significantly higher in exposed animals than controls. These data agree with the literature as well as in *D. magna* ingestion via food chain represents the principal route of toxicity. The presence of algae during exposure increased nanoparticle levels in the gut by a factor of 3. In case of direct contact with the peritrophic membrane and the cuticle, depuration is not efficient to remove nanoparticles from the organisms. In this species, the shedding of the chitinous exoskeleton is the crucial mechanism governing the release of nanoparticles regardless of the feeding regime during exposure (Auffan et al. 2013).

Furthermore, animals under fasting conditions were more exposed to nanoparticle ingestion. Nevertheless, feeding increased differences among tested dispersion/solution and controls also at T72 and T96 allowing longer exposure times; n-ZnO showed higher mortality rates than n-TiO$_2$ after T48 but this trend clearly inverted after T72-96h of exposure. These results are consistent with the literature data showing higher toxicity of n-ZnO, the higher effect due to n-TiO$_2$ over time is probably due to the minor particle size of n-TiO$_2$ nanoparticles compared to n-ZnO tested that both increased the covering effects of n-TiO$_2$ increasing mortality and immobilization rates and also a major toxicity of ingested nanoparticles of minor size compared to higher one.

Final remarks for environmental risk determination

Results reported in this study under fasting condition showed that negative controls are always within the acceptability range proposed by OECD guideline no more than T24, while, yet after 48 h of exposure under fasting conditions animals recorded effects in negative controls start to be confounding. Furthermore, in controls, effects of fasting on animal activity are notably higher compared to survival rates, this fact is consistent to the possible occurrence of precoce damages on animals' motility structures or photorecectors due to fasting condition. Jensen and Hessen (2007) reported that respiration rates were strongly affected by feeding conditions but not by food quality and feeding conditions induced higher respiration rates than fasting conditions supporting minor energy supply consumption during fasting with a reduction of activities. In this study we

performed tested on commercial nanoparticles products. Our choice was related to the fact that pristine nanoparticles are less representative of their environmental behaviour than fuctionalized ones (Nowack and Bucheli 2007).

D. magna is listed in REACH as target species to perform ecotoxicological test to evaluate environmental risks and it is standardized by the Guidelines for *Daphnia* species, acute immobilization tests (OECD 202:2004). As remarkable aspect is useful to underline as results obtained on nanoparticle toxicity by in vitro tests performed under REACH regulation assessment, required a strong standardization of exposure protocols including the production of synthetic standard ISO 6341 freshwater used to perform dissolutions not natural ones. Water chemistry is a critical key aspect that could affect toxicity of nano metal-oxides in natural water, so results obtained by in vitro tests, as required by the Law such as REACH tests, should be accurately weighted by expertise as well as standardized laboratory conditions and standardized ISO freshwater are far to natural environmental conditions. As reported in this study, the presence of feeding and/or surfactants (conditions more similar to natural environments) is able to significant affect ecotoxicological responses of exposed animals. In particular, surfactants increased toxicity of tested nano metal-oxides while feeding reduced effects observed under fasting conditions.

Furthermore, standard fasting condition represents a confounding factor due to the high energetic stress under which animals are exposed to. Energetic stress affects animals' activities and survival over time and allows realistical observations only within T24. On the other side, exposure under feeding conditions allowed to perform solid long-term observations allowing evaluating also chronic effects of tested chemicals. As reported in this study, even if animals were exposed to their LC10 for each substance, n-TiO$_2$ resulted more toxic over the time rather than n-ZnO. This fact could be due to lower particle size of TiO$_2$ compared to ZnO. Particle size (Brown et al. 2001) and specific surface area (Auffan et al., 2009) are reported to be able to affect both ecotoxicological responses and environmental behavior (Silva et al. 2014) introducing variability that should be taken into some account. Furthermore, in this study some important sources of variability under natural conditions such as pH, ionic strength of water, temperature, organic matter levels, clorine ion concentration, fulvic and humic acids levels were *a priori* standardized. Nevertheless, recent researches performed on nanoparticles produced evidences that salinity and ionic strength of water (French et al. 2009) could affect the stability of nanoparticle dispersion, sedimentation processes, and the final size and behaviour of nanoparticle aggregates (Brunelli et al. 2013; Majedi et al. 2014). Sulfur, dissolved oxygen, pH, Cl$^-$, organic compounds and lighting conditions are able to affect final toxicity of nanooxides (McShan et al. 2014) as well as

humic and fulvic acids by their complexing activity on metal ions (Dasari and Hwang 2013). The reported capability of nanoparticles to perform coating of humic/fulvic acids due to the pervasiveness of humic substances (Tang et al. 2014) shoud affect nanoparticles toxicity related to coating and effects of body burial recorded in this study. Furthermore, interactions with fulvic and humic acids could affect aggregation properties and degradation processes of nanoparticles (Tang et al. 2014; Adams et al. 2006; Quik et al. 2014) and consequently could affect the observed nets formation and ingestion rated by *D. magna* reported in this study. On the contrary effects induced by alginates in complex mixtures of nanoparticles and surfactants should be further expolored as well as alginates resulted to enhance toxicity of nanoparticles (Rottman et al. 2013).

Additionally, test methods addressing the sorption tendency of nanoparticles to algae could contribute to an improvement of the ecotox-scheme with regard to the consideration of physical effects by shading resulting in limited growth (Hund-Rinke 2018).

Conclusions

Obtained results from this study show a clear difference among ecotoxicological responses in *D. magna* exposed to nanoparticles metal-oxides under fasting standardized OECD conditions compared to feeding exposure tests. Our study supports caution when ecotoxicological data collected under in vitro standard tests are extrapolated to perform risk assessment evaluation on the environmental behaviour of metal-oxydes NPs. Furthermore, results reported in this study could provide a useful baseline to evaluate predator-prey (cladocerans-algae) interactions in aquatic environments and effects induced on the trophic web following the exposure to tested substances.

Compliance with ethical standards

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