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Chronic sublethal effects of ZnO nanoparticles on *Tigriopus fulvus* (Copepoda, Harpacticoida)

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Abstract

This study investigated for the first time the effects of ZnO nanoparticle (NP) chronic exposure (28 days) on *Tigriopus fulvus*. Acute toxicity (48 h) of three Zn chemical forms was assessed as well including the following: (a) ZnO nanoparticles (NPs), (b) Zn^{2+} from ZnO NP suspension after centrifugation (supernatant) and (c) $ZnSO_4$ H_2O. Physical-chemical and electronic microscopies were used to characterize spiked exposure media. Results showed that the dissolution of ZnO NPs was significant, with a complete dissolution at lowest test concentrations, but nano- and micro-aggregates were always present. Acute test evidenced a significant higher toxicity of Zn^{2+} and $ZnSO_4$ compared to ZnO NPs. The chronic exposure to ZnO NPs caused negative effects on the reproductive traits, i.e. brood duration, brood size and brood number at much lower concentrations (≥ 100 μg/L). The appearance of ovigerous females was delayed at higher concentrations of ZnO NPs, while the time required for offspring release and the percentage of non-viable eggs per female were significantly increased. ZnO NP subchronic exposure evidenced its ability to reduce *T. fulvus* individual reproductive fitness, suggesting that ZnO NPs use and release must be carefully monitored.

Keywords Reproductive endpoints · Chronic/acute toxicity · Copepoda · *Tigriopus fulvus* · Zinc oxide nanoparticles · $ZnSO_4$ and ionic Zn

Introduction

Nanoparticles (NPs) are currently used worldwide offering new materials and applications. Obviously, in recent years, many questions aroused on the environmental and human health risk. The knowledge about NPs impact on the environmental compartments represents a pivotal issue to promote new and safe nanotechnology in order to obtain full benefit from their applications (Espanani et al. 2015). There is still a lack of regulation on NPs use mainly due to their properties (i.e. size, shape, route of synthesis, elemental entities, surface coatings) and the physico-chemical behaviour (Nel et al. 2006). These aspects may determine undesirable toxic effects to living organisms (Papageorgiou et al. 2007; Poland et al. 2008; Oberdörster et al. 2005; Singh et al. 2007; Bernhardt et al. 2010; Faggio et al. 2018; Vajargah et al. 2018). The aquatic ecosystems are considered the major sink of NPs due to their discharge through the wastewater cycle (Nowack and Bucheli 2007; Oukarroum et al. 2012; Piccinno et al. 2012).

Among metal NPs, zinc oxide (ZnO NPs) is extensively used worldwide in several fields as electronics, medicine, feed and pharmaceutical products, ceramics, glass, cement, rubber (e.g. car tyres), lubricants, paints, ointments, plastics, sealants, pigments and personal care products

Highlights

- Acute LC50s: ZnO NPs < Zn^{2+}~$ZnSO_4$.
- Sublethal ZnO NPs caused a delay appearance of ovigerous females.
- Chronic exposure reduced nauplii per brood
- Sublethal concentration of ZnO NPs could affect the population by reducing individual reproductive fitness.

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including sunscreens, as additive in food and packaging (Gerloff et al. 2009; Nawaz et al. 2011; Piccinno et al. 2012). ZnO NPs are among the most harmful NPs to aquatic organisms (Kahru and Dubourguier 2010; Khosravi-Katuli et al. 2018). In aquatic tests, the toxicity of ZnO NPs was generally attributed to the dissolved metal form rather than the NP itself nanoforms (Franklin et al. 2007; Heinlaan et al. 2008; Mortimer et al. 2010).

Most of the currently available ecotoxicological data on ZnO NPs are focused to acute toxicity tests on freshwater organisms, including bacteria (Choi and Hu 2009; Sirelkhatim et al. 2015), algae (Franklin et al., 2007; Wong et al. 2010) and crustaceans (Heinlaan et al. 2008; Wang et al. 2009), while few studies involved marine organisms (Wong et al. 2010; Fairbairn et al. 2011; Ates et al. 2013; Manzo et al. 2013a, b; Schiavo et al. 2016). However, further studies are particularly needed for a proper risk assessment in seawater, where the high ionic strength and pH conditions are known to alter the NP physico-chemical properties and toxicity (Wong et al. 2010; Rotini et al. 2017, 2018).

It is important to highlight that there is a considerable lack of information on ZnO NP chronic effects, although the assessment of chronic toxicity is essential for environmental ecotoxicological studies since they can be predictive of population-level effects (Wong et al. 2010; Peng et al. 2011; Ates et al. 2013; Hanna et al. 2013; Keller et al. 2013, 2014; Ma et al. 2013; Manzo et al. 2013a, b). The chronic exposure, if compared to the acute one, has a greater ecological relevance, increased sensitivity, better prediction of toxicity and effects on population dynamics (USEPA/USACE 2001; Scarlett et al. 2007; Greenstein et al. 2008). Thus, chronic toxicity assessments such as life cycle studies are needed to improve our understanding of the long-term and low-dose effect of these nanoparticles, particularly in the marine environment.

Harpacticoid copepods play an important role as a link in aquatic food chains, ranging from phytoplanktonic algae or members of the microbial loop to secondary consumers (e.g. fishes) (Raisuddin et al. 2007). Among them, *Tigriopus fulvus* is an abundant marine species and is a well-established test species in acute toxicity testing. It offers distinct advantages, as it is easily cultured under laboratory conditions, has year-round availability, good sensitivity to different toxicants and good reproducibility of tests (Faraponova et al. 2005, 2016; Mariani et al. 2006; Tornambè et al. 2012; Prato et al. 2011; Prato et al. 2012, 2013, 2015). Further, it is suitable for use in the chronic tests (short life cycle, high offspring production), allowing assessment of endpoints related to larval development and reproduction.

To the best of our knowledge, the acute and chronic ecotoxicity of ZnO NPs on marine copepods is still unexplored, except for a preliminary study on the same species (Parlapiano et al. 2017). The aim of this study is to investigate the acute and chronic ecotoxicity of ZnO NPs evaluating critical life cycle traits of *T. fulvus*. Moult number, developmental times of nauplius and copepodite phases, fecundity and hatching time were chosen as sublethal endpoints in chronic exposure and survival in acute exposure tests.

Materials and methods

Test organism culturing

Tigriopus fulvus was obtained from a massive culture maintained in the CNR-Institute for Coastal Marine Environment of Taranto. The cultures were kept in 0.5-L polystyrene flasks with filter screw caps containing natural sea water (NSW, 0.22-μm cellulose membranes) and a salinity of 38‰ under a light:dark cycle of 18:8 h in a temperature-controlled room at 20 °C (UNICHIM 2396 2014). Copepods were fed weekly ad libitum with Tetramarin (fish food) and a mix of microalgae including *Tetraselmis suecica* and *Isochrysis galbana*. These algae were cultured in a temperature-controlled room, using 500-mL flasks filled with autoclaved NSW, collected in an unpolluted area and filtered through a GF/C Wathman 0.22-μm filter (Prato et al. 2015).

The experiments were performed in three replicates on neonate (nauplii) originating from synchronized cultures (24 h). Nauplii were released by ovigerous females selected 24 h prior the test, transferred on 80 μm mesh plankton net fixed to a Plexiglas tube and fed with a mix of *T. suecica* and *I. galbana* algae cultures at 1.5×10^8 and 3.0×10^8 cells/L density, respectively.

Ecotoxicity

Experimental design of this study included two steps: (i) Comparison of acute toxicity of ZnO NP suspension with the acute toxicity of Zn^{2+} and $ZnSO_4$ on mortality of *T. fulvus* and (ii) evaluation of chronic effects of ZnO NP on *T. fulvus* life cycle.

During the entire duration of all toxicity tests, pH and salinity of the test solutions remained almost stable; pH ranged from 8.1 to 7.8 and did not vary by more than 0.2 units in any given test, while salinity was 38 ± 1 ‰. Oxygen levels in all toxicity tests were above 80% of saturation, meeting the validity criteria set by the OECD guidelines (2004).

Acute toxicity test (48 h)

The experiment consisted in exposing *T. fulvus* nauplii (≤ 24 h-old) for 48 h at increasing concentrations of Zn in three forms: (a) ZnO NP suspension; (b) Zn^{2+} from ZnO NP suspension after centrifugation (supernatant); and (c) $ZnSO_4$, in static acute tests with mortality as endpoint. A negative

control (without Zn, only NSW) was included in the experimental design. The Zn concentrations ranged from 0.5, 1, 2, 4 and 8 mg/L.

Before the tests, about 40 nauplii were transferred to a Petri dish containing one of the concentrations to test, avoiding dilution in the final test concentrations. The tests were performed in sterile 12-well multi-plates (5 mL per well). Ten nauplii were randomly chosen from each Petri dish and transferred to a well containing 3 mL of the test suspension/solution or NSW. The experiment was carried out in triplicate; any food was administered to the animals for the entire duration of the test.

Mortality of copepods was checked under a stereomicroscope. Copepods were considered dead if any movement of external appendages was registered for about 20′ in response to mechanical stimulation.

Oxygen content and pH of the test media were measured at the beginning and at the end of each test in one replicate of both control and test concentrations.

Chronic toxicity test (28 days)

Semi-static renewal toxicity tests to evaluate the larval development and reproductive traits were conducted with newly hatched nauplii (≤ 24 h old) of *T. fulvus* exposed for 28 days at increasing concentrations of ZnO NPs looking for lethality, nauplii/copepodites ratio (after 5 days), ovigerous females appearance time, hatching time and mean brood per female, aborted egg sacs and not developed eggs. Test concentrations were selected based on values lower than the NOEC acute test (0.05, 0.10, 0.15, 0.20 and 0.25 mg/L) including a negative control with NSW. Three replicates were performed for each concentration and control, and the assay was carried out for three times consecutively. Salinity, light and temperature conditions are those described for massive culture: salinity 38‰, light:dark cycle of 18:8 h and temperature 20 °C.

Practically, females with egg sacs were selected and kept one per Petri dish (Sterilin, UK), containing 25 mL NSW. The selected females were fed with live algae (*T. suecica* and *I. galbana* of 5×10^8 cells/mL). After 24 h, newly hatched nauplii were collected in a new Petri dish, containing 25 mL fresh NSW. Twenty-four healthy nauplii (i.e. actively swimming) were randomly selected, and tested separately as replicates, in 3-mL multiwell plate containing 1 mL of test solution and algal cells (*T. suecica* and *I. galbana* of 5×10^8 cells/mL) as food supply. Test solutions with food supply were renewed every second day; all the copepod batches were transferred into a new multiwell plate.

Daily, the survival of each copepod was checked and recorded in all wells, under the stereomicroscope. Concurrently, the development stage was determined and the number of nauplii and copepodite moults was counted to calculate the duration of the naupliar and copepodite stages and the time taken to reach adulthood. The degree of larval development in each replicate was expressed as the ratio of copepodites to the total number of organisms (i.e. the sum of nauplii and copepodites); the results were reported as percentage of moults, at each concentration.

Nauplii survival rate was calculated as the percentage of survived nauplii reaching the copepodite stage; adult survival rate was calculated as the percentage of surviving copepods (male and female) after 28 days.

In order to determine reproductive parameters, soon after the juvenile's phase, male and female copepods were placed in the same well to allow mating. The males were eliminated from the experimental wells after the mating because *T. fulvus* females require only one mating to fertilize all the eggs produced during their entire life span. Twelve females, with the egg sac, were transferred and monitored in isolation (one per well), in each ZnO NP concentration, until the offspring release. Daily, each test well was checked for female mortality and offspring presence and numerosity. Every 48 h, females were transferred to a new culture plate with newly prepared solutions and feed. Newborn nauplii were counted and removed from the test chambers daily, since the appearance of the first brood. Fecundity (offspring production) was assessed as number of nauplii per female. The number of broods and nauplii/brood was quantified for each female. To determine the hatching success, aborted egg sacs and not developed eggs were also enumerated.

Preparation of testing solutions/suspensions

Zinc oxide NPs were purchased from US Research Nanomaterials, Inc. as a water dispersion (20 wt%, purity of 99.95%) with a nominal particle size in the range of 30–40 nm. ZnO NP stock suspension (200 mg/L) was prepared in 0.22 μm filtered Milli-Q water from the 20% dispersion and sonicated for 15′ in a sonicator bath (305 W, 50–60 Hz; Soltec Ultrasonic Baths) and then stored in the dark at 4 °C.

The final testing suspensions were prepared from ZnO NP stock after 15′ sonication, using natural seawater (NSW) collected from the Mar Grande of Taranto (Ionian Sea, Italy; pH 8.0 ± 0.1, salinity 38 ± 1 and 0.22 μm filtered). For the acute test, the final nominal concentrations were 0.5–1.0–2.0–4.0–8.0 mg/L. In addition, an aliquot (30 mL) of each final suspension was first centrifuged (13,000×*g* for 20′) and filtered (0.22-μm filter) to remove ZnO NPs and their aggregates. The final nominal concentrations of ZnO NP suspensions for the chronic test were 0, 0.05, 0.1, 0.15, 0.2 and 0.25 mg/L, chosen on the base of acute toxicity test results.

To discriminate the acute toxicity due to the Zn dissolved from NPs from that due to the NP suspensions, a dedicated test with the supernatant of the centrifuged suspensions 16,000 *g* for 30 min -Bekman Coulter™ Avanti J-301 centrifuge) was also carried out. The concentrations of centrifuged

suspensions were the same as reported above. The stock solution of $ZnSO_4$ (Sigma-Aldrich) was prepared in 0.22-μm filtered Milli-Q water (mQW); stirring the solution until metal was completely dissolved. The final nominal test concentrations were prepared in NSW at 0.5, 1.0, 2.0, 4.0 and 8.0 mg/L for the acute tests.

Particle size distribution, stability of Zn solutions/suspensions and Zn determination

The size distribution of the Zn solution/suspension in filtered NSW were obtained by using a dynamic light scattering (DLS) composed by a Photocor compact goniometer, a SMD 6000 Laser Quantum 50 mW light source operating at 5325 Å and a PMT-120-OP/B and correlator (Flex02-01D) purchased from Correlator.com. From the diffusion coefficient obtained from the fitting of the correlation function and in the approximation of diluted solution, the hydrodynamic radius was evaluated through the Stokes-Einstein equation Mangiapia et al. 2013). Each measurement was performed in triplicate. The samples (1 mg/L) were prepared from a starting solution (1 g/L) produced with 0.22 μm filtered ultra-pure water in 0.22-μm filtered NSW and sonicated at 100 W for three cycles of 5 min each leaving to rest the sample for 5 min between two cycles. Finally, each sample was left to equilibrate in the instrument for 15 min before the measurement.

Only for ZnO NPs, size distribution was followed from starting time (i.e. 15 min after sonication—same conditions previously described) up to 48 h of ageing in filtered NSW.

The size of the ZnO NPs (from the purchaser) was evaluated by mean of transmission electron microscopy (TEM) by using a Philips EM 208S with an accelerating voltage of 100 kV.

The concentration of Zn from Zn^{2+}, $ZnSO_4$ and ZnO NP solution/suspensions was quantified by Inductively Coupled Plasma Mass Spectrometry (Aurora M90 Bruker, USA) (Table 1). The detection limit (LOD) and limit of quantification (LOQ) were calculated using the method of blank variability for each investigated metal (EPA 6020b 2014).

Statistical analysis

All data are presented as mean and the relative standard deviation (SD). For acute toxicity tests, lethal concentrations that resulted in 50% mortality were chosen as the endpoint. Data were analysed for normality and variance homogeneity through Kolmogorov–Smirnov and Bartlett's tests, respectively. The LC_{50} values of the 48 h exposure were calculated according to nonlinear multiple regressions (Sigmoidal four parameters) with Sigma Plot 11.0. One-way ANOVA analyses were used to determine statistical significance of the differences among treatments and between all treatment levels and the control for all the endpoints ($p < 0.05$). If significant differences were found by the ANOVA, Tukey multiple comparison test was used to discriminate between the means. Data are presented as the means ± standard deviation (S.D.). All statistical analyses were conducted using Past3 Version 1.0 software.

Results

Characterization of NPs

The analysis of 1 mg/L of ZnO suspension from the purchaser in filtered NSW (15 min sonication and 15 min ageing) evidenced that nanoparticles ranged between 10 and 20 nm (Fig. 1). The hydrodynamic radius distribution of NPs was investigated in all solution/suspension (Zn^{2+}, $ZnSO_4$ and ZnO NPs) at 1 mg/L after 1 h from their preparation (including 15 min sonication). Most aggregates in Zn^{2+} and $ZnSO_4$ solutions ranged approximately around 770 ± 40 nm and 950 ± 50 nm, in that order. In ZnO NPs, aggregates presented a bimodal trend showing two main distribution peaks (150 ± 20 nm and 640 ± 30 nm) (Figure S1). Zn^{2+} solution presented aggregated probably due to resuspension during sampling and/or colloids' formation. After 48 h of ageing (Figure S2), the ZnO NP aggregates presented a unimodal distribution with a mean hydrodynamic radius of 140 ± 20 nm that was similar to that at zero time (110 ± 20 nm), suggesting that during the exposure period the hydrodynamic radius of aggregates stabilized, and the peak at 640 ± 30 nm completely disappeared. Thus, specific changes in the hydrodynamic radius can occur in solution/suspension according to the type of Zn and contact time both in nano- and no-nano spiked media.

Acute toxicity test

In all acute toxicity tests, the survival of the controls was > 98% as required by OECD guideline 202 (OECD 2004).

Table 1 Measured concentrations as total Zn; Zn^{2+} in the supernatant of the centrifuged suspensions (Zn^{2+}), $ZnSO_4$ and ZnO NP in final testing solutions/suspensions; mean ± standard deviation of measurements. FNSW = filtered natural saltwater

	Total Zn (mg/L)		
FNSW	0.014 ± 0.003		
Testing concentrations	Zn^{2+}	$ZnSO_4$	ZnO NPs
1	0.014 ± 0.003	0.52 ± 0.10	0.48 ± 0.09
2	0.54 ± 0.11	0.85 ± 0.17	0.85 ± 0.17
3	0.86 ± 0.17	1.73 ± 0.35	1.26 ± 0.25
4	0.96 ± 0.19	3.89 ± 0.78	4.31 ± 0.86
5		7.95 ± 1.59	4.25 ± 0.85

In all exposure scenarios (Zn^{2+}, $ZnSO_4$ and ZnO NPs), mortality showed a concentration-response relationship reaching 100% effect after 48 h at the highest exposure concentrations. Statistical analysis showed no significant differences between *T. fulvus* exposed to Zn^{2+} and $ZnSO_4$ scenarios ($p < 0.05$), while LC50 decreased approximately of 30% with ZnO NP. As reported in Fig. 2, LC_{50} values were 0.93 (0.84–1.02) mg/L, 0.85 (0.81–0.89) mg/L and 1.27 (1.15–1.40) mg/L for Zn^{2+}, $ZnSO_4$ and ZnO NPs, respectively. Statistical analysis highlighted the following significant differences: ZnO NPs < $ZnSO_4$~Zn^{2+} (ANOVA, $p < 0.05$, *post-hoc* Tukey's test). The sensitivity of *T. fulvus* was higher than *T. japonicus* of approximately five times (LC50 = 5.8 (5.05–6.64) mg/L) (Yi et al. 2019).

Chronic toxicity test

No inhibitory or stimulatory effect on the larval development of *T. fulvus* exposed to ZnO NPs was detected up to 0.25 mg/L, the highest tested concentration. After 5-day exposure, the percentage of nauplii developed to copepodites ranged from 82% (0.20 and 0.25 mg ZnO-NP/L) to 94% at 0.15 mg ZnO-NP/L (Fig. 3).

After 28-day exposure, the survival of *T. fulvus* was very high (from 95 to 100%), with no significant differences between control and all treatment groups (ANOVA, n.s.).

Females became ovigerous around day 11; 1-day delay was observed at the highest concentrations (0.20 and 0.25 mg/L ZnO-NP; ANOVA, $p < 0.05$) (Fig. 4a). The production of the egg sacs occurred 24 h after the pairing, without differences among treated and control animals (ANOVA, n.s.). A significant delay in the time required for the offspring release was found hatching took 2.58 ± 0.17 days in the control batches and 2.93 ± 0.10 and 2.95 ± 0.26 days at 0.20 and 0.25 mg/L (ANOVA, $p < 0.05$) (Fig. 4b). A reduced number of broods per female were also recorded at all ZnO NP concentrations, ranging from 6.7 ± 0.2 in control to 2.8 ± 0.3 at 0.25 mg/L (ANOVA, $p < 0.05$; Fig. 4c); significant differences were also found between the 0.25 mg/L ZnO NP batch and those exposed to lower concentrations (ANOVA, $p < 0.05$).

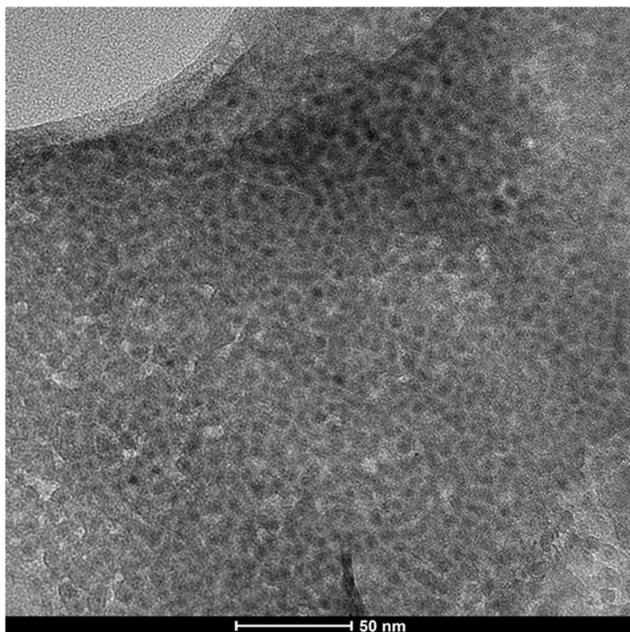


Fig. 1 TEM picture of ZnO NPs (1 mg/L suspension from the purchaser in filtered NSW—15 min sonication and 15 min ageing)

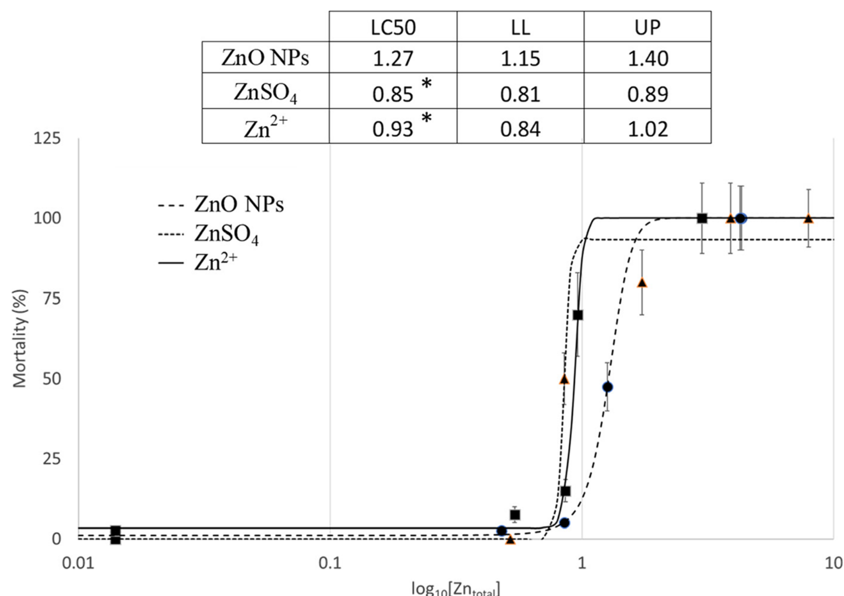
The mean number of nauplii per brood was significantly reduced under ZnO NP exposure (ANOVA, $p < 0.05$; Fig. 5a), ranging from 5325 ± 3 at 0.05 to 1010 ± 1 at 0.25 mg/L of ZnO NPs. Consequently, fecundity (as mean total number of nauplii per female at day 28; Fig. 5b) was significantly reduced if compared to control (ANOVA; $p < 0.05$), ranging from 164 ± 11 in control to 727 ± 5 at 0.25 mg/L of ZnO NP. Non-viable eggs per female increased with ZnO NP concentration (Fig. 5c), from 25.4% at 0.15 mg/L to a maximum of 68% at 0.25 mg/L.

Discussion

The bioassays with *T. fulvus* successfully assess both acute and chronic toxicity of ZnO NPs and their characterization in the saline test medium, as requested by the OECD guidelines (2004) to superimpose the two set of data for the correct interpretation of the ecotoxicological results.

Characterization of NPs

The aggregation and stability of ZnO NP suspensions were measured in NSW. NSW background particles content was substantially not detectable probably due to the initial 0.22-μm NSW filtration. After 48 h, aggregates slightly increased compared to zero time, passing from 110 ± 20 nm up to 140 ± 20 nm (Figure S2). In Zn^{2+} and $ZnSO_4$, aggregates of different sizes are present (Figure S1), suggesting that solution/suspension contained nano-(micro-)sized fractions potentially able to interact with biota. This was in contrast with other studies where ZnO NP dissolution was very rapid demonstrating that dissolution of ZnO NPs happened in the first hours, soon reaching 50–65% of the initial NP load, with no differences in the ionic Zn released into seawater by ZnO NPs between 24 and 48 h (Schiavo et al. 2018; Oliviero et al. 2017). Overall, our results confirmed that free Zn^{2+} concentration in the exposure medium depends on the NP concentration in the suspension and on their size and exposure time as already suggested in previous studies (Ates et al. 2013; Wang et al. 2009).



	LC50	LL	UP
ZnO NPs	1.27	1.15	1.40
$ZnSO_4$	0.85 *	0.81	0.89
Zn^{2+}	0.93 *	0.84	1.02

Fig. 2 Acute static test (48 h) results as median lethal concentration (LC50) of *T. fulvus* exposed to the ZnO NP, $ZnSO_4$ and Zn^{2+} suspension/solutions expressed as total Zn (mg/L). LL lower limit, UP upper limit; asterisk = not significantly different ($p < 0.05$, Tuckey's test)

Acute toxicity test

The three different Zn scenarios induced significant differences among acute toxicity responses in *T. fulvus*: ZnO NPs and $ZnSO_4$ < Zn^{2+}. This result mainly depends on NP dissolution, which play a crucial role in ZnO NP toxicity as already demonstrated in other studies (Matranga and Corsi 2012; Manzo et al. 2013a, b. Zn^{2+} dissolution from NPs is considered the major driver of toxicity (Franklin et al. 2007; Blinova et al. 2010; Buerki-Thurnherr et al. 2013; Ma et al. 2013), causing cytotoxicity (Xia et al. 2008), disruption of cellular zinc homeostasis, lysosomal and mitochondrial damage, and cell death (Franklin et al., 2007). Even though, other studies suggest that Zn^{2+} cannot account entirely for the toxicity of ZnO NPs (Wang et al. 2009; Poynton et al. 2011; Li et al. 2018) and it is still not clearly understood to which extent the dissolved Zn^{2+} can contribute to ZnO NP toxicity and what are the mechanisms involved (Ma et al. 2013). Baun et al. (2008) proposed that the toxicity of ZnO NPs to could be due to their adhesion to the crustaceans exoskeleton, causing a mobility reduction or even loss.

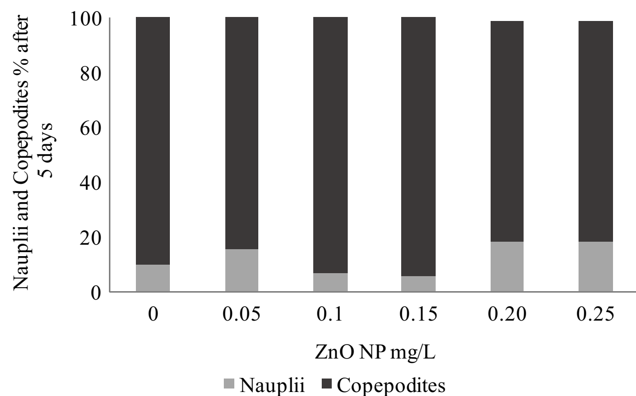


Fig. 3 Nauplii and copepodites percentage (%) of *Tigriopus fulvus* exposed for 5 days to ZnO NP different test concentrations

The results of our experiments, comparing the toxicity of ZnO NP suspensions, before and after the removal of NPs by centrifugation, clearly showed that supernatants without NPs have a higher toxicity. This could be ascribed to the removal of NPs, increasing the progressive amount of dissolved zinc in the exposure media. These results strongly support the importance of comparing the suspension vs supernatant toxicity to go into the deep of the specific toxic activity of nanosized particles.

In this study, the acute toxicity results are comparable with those found in the literature for both ZnO NPs and $ZnSO_4$ and Zn^{2+}. Wong et al. (2010) reported for *Tigriopus japonicus* a 96-h LC_{50} of 0.85 mg/L for ZnO NPs and 1.14 mg/L for $ZnSO_4$ $7H_2O$, and for *Elasmopus rapax*, a 96-h LC_{50} of 1.19 mg/L for ZnO NPs and 0.80 mg/L for $ZnSO_4$ $7H_2O$. Park et al. (2014) for *T. japonicus* reported a value of 96 h LC_{50} of 2.44 mg/L of ZnO NPs. All the data are within the same order of magnitude and differences are expected among NP ecotoxicological studies, due both to differences in the experimental procedures, especially concerning preparation and/or suspending of NPs (presence/absence of solubilization vehicles, filtering, centrifugation, sonication), and to the nature of nanomaterials, making comparisons quite hard (Schrurs and Lison 2012).

Chronic toxicity test

The present study, for the first time, thoroughly investigated the chronic toxicity of ZnO NPs in *T. fulvus* by evaluating the sensitivity of sublethal endpoints in this copepod species, as no information can be found in the literature, except for

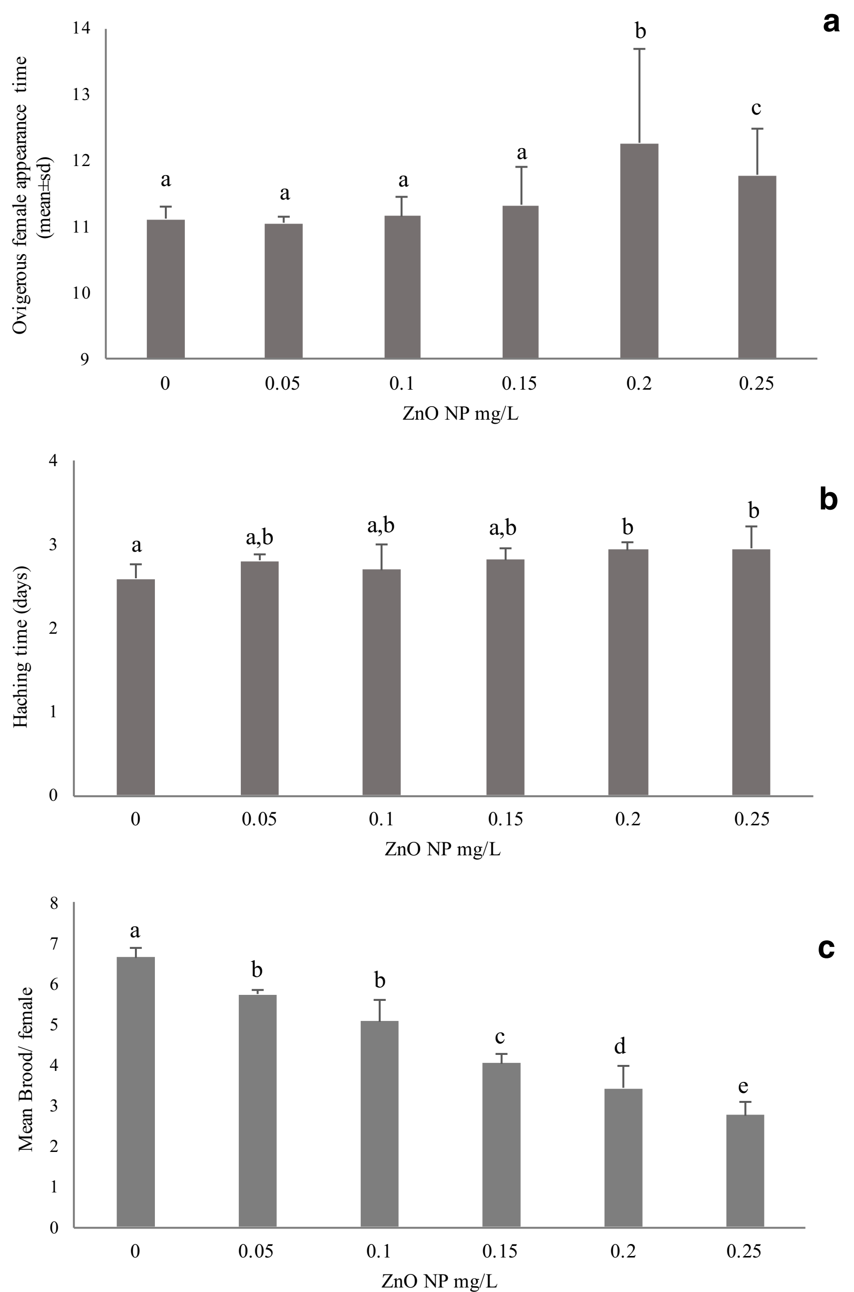


Fig. 4 Ovigerous female ($n = 20$) appearance time (days) (**a**), hatching time (days) (**b**), mean brood/female (**c**) of *T. fulvus* exposed to ZnO NP final suspensions. Data with different superscript letters significantly differ ($p < 0.05$)

Parlapiano et al. (2017) already performed within part of the current research group. The organism exposure from hatching to reproductive maturity allows observing several aspects of embryonic/larval development along with reproductive parameters. To this end, the distinct developmental stages of *Tigriopus* genus provide a well-defined set of endpoints to be used in ecotoxicity tests, which allow an easy tracking of development (Kwok et al. 2015; Biandolino et al. 2018). The chronic exposure to ZnO NPs did not significantly affect the developmental time taken by nauplii to reach the copepodite or adult stage, while it caused clear negative effects on the reproductive traits, such as brood duration, brood size and brood number. Even the timing of phenological events (e.g. the appearance of ovigerous females and the time required for the offspring to be released) showed significant delay at the highest concentrations of ZnO NP, showing to be sensitive endpoints easy detectable in short times. The most affected reproductive endpoint was fecundity with a significant reduction of nauplii number produced per female during the 28-day exposure, even at low concentrations.

Sublethal tests (and endpoints) are widely used to improve the prediction of chronic effects, difficult to achieve with acute lethality tests (Barata et al. 2002), being accepted that lethal endpoint cannot accurately assess the effect of

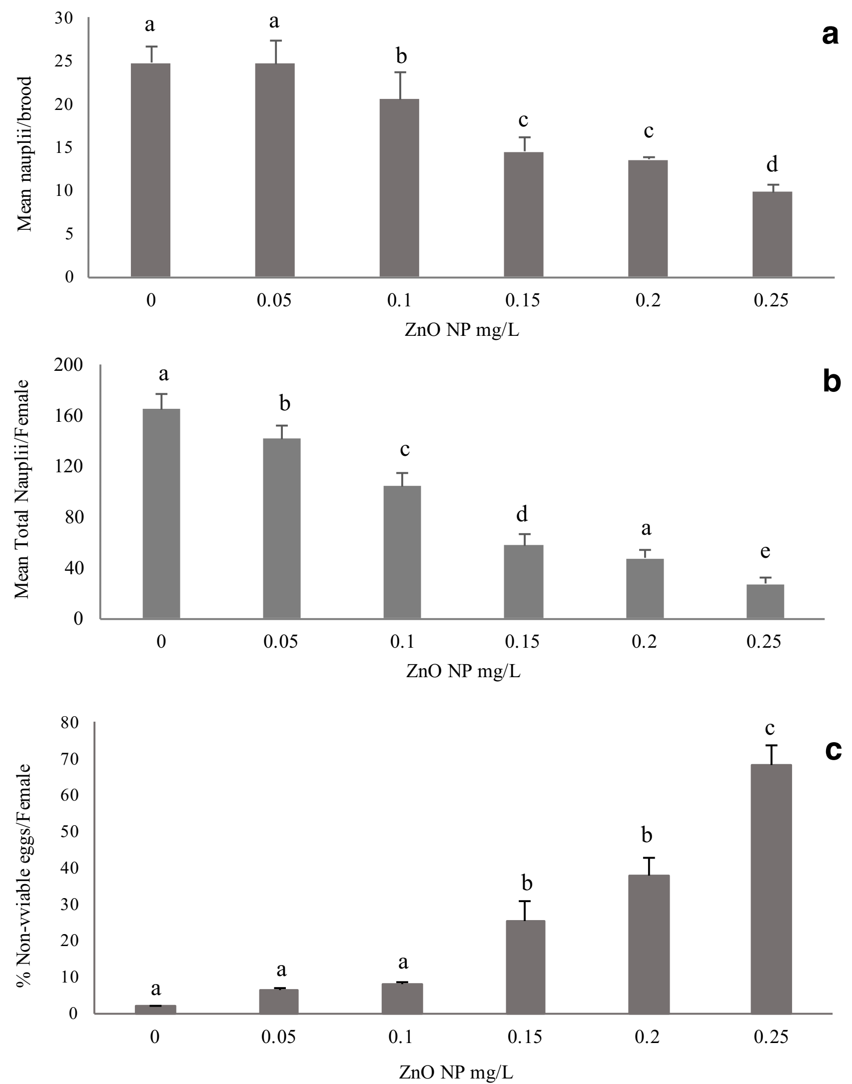


Fig. 5 Mean nauplii/brood (**a**), mean nauplii/female (**b**) and non-viable eggs/female (%) (**c**) at the end of the experiments (on day 28) of *T. fulvus* exposed to ZnO NP final suspensions. Data with different letters differ significantly ($p < 0.05$)

toxicants (Cairns 1992). Chronic toxicity tests are recommended in risk assessment, especially when the mode of action of the toxicant is yet not well established (Ingersoll et al. 1999), as in the case of NPs. For NPs, it is still necessary to identify and select the most useful endpoints to define their toxicity. In this study, the chronic exposure to ZnO NPs did not impair the time taken by nauplii to develop into copepodites or adults; thus, this endpoint can be considered as not useful to detect ZnO NPs chronic effects of and can be excluded by the testing investigations. Probably, ZnO NPs is not involved in the processes of moulting and metamorphosis, controlled by ecdysteroids or other hormones (Laufer and Borst 1988). The chronic exposure to ZnO NPs impaired more the reproductive traits (brood duration, size and number) and the timing of phenological events. Hence, both endpoints are sensitive and worth to be included in ZnO NPs investigation panel. In this case, ZnO NPs can delay nauplii hatching delay by affecting some enzymes, such as the proteolytic ones (i.e. zinc-proteases), involved in many physiological processes (cell migration, tissue repair, etc.), as happens in fish larvae (Inohaya et al. 1997). In addition, ZnO NP aggregates, increasing in size at increasing NP concentration, might occlude the chorion channel pores reducing oxygenation, essential for nauplii development, as reported for fish (Cheng et al. 2007). The most promising reproductive endpoint is fecundity, as evidenced by the significant results, even at low concentrations. ZnO NPs affect number of broods/females in inverse relation with exposure and this effect may be due to the dissolved Zn^{2+}, a potential disruptor of calcium homeostasis during the first developmental phases (Warnau et al. 1996). Finally, the egg viability is also an interesting and sensitive endpoint; ZnO NPs reduced egg viability up to about the 70%, suggesting that Zn can have a direct lethal effect on the developing embryos. Comparable data were obtained by Huang et al. (2017) testing ZnO NP chronic exposure of the nematode *Caenorhabditis elegans* showing a significant inhibition of growth and reproductive

capability. Lagido et al. (2009) ascribed ZnO NP toxicity to the reduction of ATP production consequent to the heavy metal detoxification mechanisms and to the increased ROS levels, being an extra energetic cost affecting mitochondria function. The ability to generate ROS, leading to oxidative stress and damages to lipids, carbohydrates, proteins and DNA leading to cell death is a relevant adverse effect of NPs (Kohen and Nyska 2002; Fulda et al. 2010). Hence, the exposure to sublethal Zn concentrations could potentially affect the population dynamics by reducing individual reproductive fitness and overall reproductive output. These results are consistent with Fabrega et al. (2011) that reported that Zn exposure both as ZnO NP, Zn^{2+} and bulk ZnO (in the range of 0.2–1.0 mg/L) delayed growth affecting the reproductive outcome of the amphipod *Corophium volutator*. Garbutt and Little (2014) reported that ZnO NPs affect both the number of offspring and the feeding rate of the cladoceran *Daphnia magna*.

Moreover, the discussion remained open about the influence of microalgae as feed supplement added to testing solutions/suspensions during the exposure scenarios. *T. fulvus*, as already reported by Jarvis et al. (2013), could be overexposed to NPs that can adhere to or enter the algal cells constituting a supplemental indirect dietary source.

Conclusions

This study demonstrated the toxic effects of ZnO NPs to *T. fulvus* in both acute (48 h) and chronic assays (28 days). To the best of our knowledge, this is the first study that extensively explored the chronic effects of ZnO NP exposure on the reproductive traits of *T. fulvus*. The results of this study highlighted (i) the potential environmental risk due to the presence of ZnO NPs in marine environments, even at low concentrations, and (ii) the suitability of the chronic bioassay for the evaluation of both conventional contaminant (Zn^{2+} and $ZnSO_4$) and ZnO NPs. Toxic effects of ZnO NPs were evidenced from 100 μg/L onwards. These concentrations are similar to the detected environmental ones, and forecasts suggested a further increase in ZnO NPs use and subsequent release in the near future.

Given the high abundance of copepods in the marine environment and their important role in the carbon cycle and food webs, the comprehension of the impact of emerging pollutants on these organisms is essential. In the marine systems, the reduction of an important prey population (i.e. *T. fulvus*) could trigger a bottom-up cascade impact on both the food web and the energy flow, leading to a possible change in the marine benthic community composition and structure in the long run.

Finally, the balance between the usefulness of ZnO NPs and the risks for living organisms deserves a constant monitoring to avoid repercussions on wildlife and, ultimately, to human health.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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