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Effects of nickel oxide nanoparticles on survival, reproduction, and oxidative stress biomarkers in the marine calanoid copepod Centropages ponticus under short-term exposure

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Abstract :

Excessive use of nickel oxide nanoparticles (NiO NPs) in various industrial and commercial products can lead to various negative effects in human and environmental health due to their possible discharge into the environment. Nerveless, information about their ecotoxicological effects on marine organisms are lacking. Copepods are good ecotoxicological models because of their high sensitivity to environmental stress and their key role in the marine food webs. In this study, 48 h acute tests were conducted on the marine planktonic copepod Centropages ponticus to assess lethal and sublethal toxicities of NiO NPs. The results revealed LC50 (48 h) of 4 mg/L for adult females. Aggregation and settling of NiO NPs were observed at concentrations ≥ 2 mg/L. Exposure to sublethal concentrations (≥ 0.02 mg/L for 48 h) had significant negative effects on reproductive success in C. ponticus. Egg production after 24 h and 48 h decreased by 32% and 46%, respectively at 0.02 mg/L and 70% and 82%, respectively, at 2 mg/L. Hatching success was reduced by 70% and 79% at 2 mg/L for eggs produced after 24 h and 48 h respectively. Antioxidant enzymatic activity increased significantly with NiO NP concentration and time, indicating that NiO NPs can cause oxidative stress in C. ponticus even under short-term exposure, while significant inhibition of acetylcholinesterase activity at 2 mg/L after 48 h suggests neurotoxic effects of NiO NPs.

Keywords : Metal oxide nanoparticle, Copepods, Acute toxicity test, Egg production, Egg hatching success, Antioxidant enzymes

Introduction

Nanoparticles (NPs) represent a serious environmental threat due to their toxicity and persistence (Geissen et al. 2015). According to the British Standards Institution, these compounds are defined as discrete pieces of material with all three external dimensions in the nanoscale (size <100 nm). Size, shape, chemical composition and structure of particle are main factors which determine the nanoparticle properties (optical, electrical, and magnetic properties) (Khan et al. 2019). Based on their chemical composition, NPs can be divided into various groups: *e.g.*, metallic, polymeric metallic oxide, semiconductor, and metallic oxide nanoparticles. Among the metallic oxide group, nickel oxide nanoparticles (NiO NPs) have widespread use in many industrial and commercial products (*e.g.*, solar cells, lithium-ion batteries, resistive random-access memory, and biosensors), due to their magnetic properties (Tadik et al. 2015). There are also multiple medical and biotechnological applications of NiO NPs, such as remediation of heavy metal contaminated water and enhancement of biogas production from macrophytes of wastewater treatment plants (Mahmoud et al. 2015; Salama et al. 2020). A recent study has reported an annual production of NiO NPs of 20 tons only in the United states (Gomes et al. 2019) which increases progressively the levels of NiO NPs exposure as stated by Avila-Arias et al. (2019). The excessive use of NiO NPs leads to their possible widespread distribution in terrestrial, estuarine, freshwater, and marine ecosystems through industrial and domestic wastewaters and aerial deposition (Wiesner et al. 2006). Despite the number of studies on NiO NPs, the environmental concentrations and distribution in field environmental matrices (water, sediment, and biota) have not yet been explored. Research investigating environmental nanoparticle concentrations in aquatic ecosystems has shown that these compounds are detectable in aquatic matrices at concentrations up to µg/L (Zhang et al. 2019). Overall, the behavior of nanoparticles varies with the environmental matrix (water, sediment, soil, etc.). For NiO NPs, aggregation behavior in seawater has been observed due to their low solubility and high stability (Gong et al. 2016; Oukarroum et al. 2017).

There are numerous concerns about the risks posed by nanoparticle pollution to the environment and human health (Ates et al. 2016; Oukarroum et al. 2017). Previous laboratory studies have shown that NiO NPs have the potential to cross biological membranes and to accumulate in target tissues, causing harmful toxic effects (Ates et

al. 2016; Oukarroum et al. 2017). In addition, Ates et al. (2016) showed that NiO NP accumulation increases with exposure concentration and duration. Acute and chronic exposures to NiO NPs have been associated with mortality effects on aquatic organisms, depending on species sensitivity (Kovrižnych et al. 2014; Oukarroum et al. 2017). NiO NPs can limit cell division, as observed for the green algae *Chlorella vulgaris*, and can also limit reproduction in zebrafish (*Danio rerio*) (Oukarroum et al. 2017). Moreover, NiO NPs have been shown to be associated with cytotoxic and genotoxic effects even under acute exposure in algae, rats, and human cells (Ada et al. 2010; Oukarroum et al. 2017). The toxicity of nanoparticles is influenced by their intrinsic characteristics (size, surface area, chemical composition, crystal structure, shape, etc.) and their behavior (aggregation, dissolution) (Sukhanova et al. 2018). Studies examining the toxicity mechanism of NiO NPs have found that they cause toxicity in aquatic organisms by induction of oxidative stress through generation of high levels of reactive oxygen species (*i.e.*, hydrogen peroxide: H_2O_2, the superoxide anion: O^{2-}, and the hydroxyl radical: OH) and disruption of antioxidant enzymes such as glutathione (GSH), as observed in algal cells (Manke et al. 2013; Oukarroum et al. 2017). Disruption of the antioxidant system can be associated with major risks, such as neurotoxicity, lipid peroxidation, and apoptosis (Ada et al. 2010; Ates et al. 2016).

Despite the known toxicity of NiO NPs, marine species have received little attention, as noted by Gong et al. (2019), in comparison with freshwater species (Oukarroum et al. 2017; Sousa et al. 2018a, b). Thus, ecotoxicological studies of the effects of NiO NPs on marine organisms are needed. Copepods may be interesting marine biological models for such studies, considering their essential role in marine ecosystems as main secondary producers in food webs and as vital links between phytoplankton and higher trophic levels, to which they transfer carbon and energy (Jayalakshmi and Santhanam 2019). In addition, copepods can represent a pathway for contaminant entry for higher trophic links (Lauer and Bianchini 2010; Cailleaud et al. 2011). Moreover, copepods are known as good bio-indicators of ecosystem pollution, because of their sensitivity to several environmental disturbances (Bianchi et al. 2003; Hussain et al. 2020). These organisms can develop adaptive responses to oxidative stress using a sophisticated biochemical defense mechanism (Kim et al. 2014). The marine calanoid copepod *Centropages ponticus* is a cosmopolite species and has been used previously as an experimental model for ecotoxicological tests due to its short

123 life cycle, its small size, and its high sensitivity to contaminants such as metals
124 (Ensibi et al. 2015; Ensibi and Yahia 2017). In this study, we conducted acute toxicity
125 tests to investigate the lethal and sublethal effects of NiO NPs on *C. ponticus* under
126 short-term exposure. Indeed, acute tests mimic the effects of NPs under accidental and
127 short-term release into the environment. In addition, due to their lifestyle and ability
128 to swim, planktonic organisms, like copepods, should experience shorter time
129 exposure than benthic organisms for example. Moreover, due to their rapid
130 transformation behavior, NPs, such as NiO NPs, aggregate in marine waters and
131 deposit in the sediment which reduces their bioavailability for aquatic pelagic
132 organisms and raises the risk for benthic organisms (Miglietta et al. 2015). Finally,
133 results from acute tests allow selecting a range of NiO NPs concentrations to be used
134 in sublethal tests. Copepod reproductive traits and molecular responses (*e.g.*, egg
135 production rate, hatching success, nauplius survival rate, biomarkers, and genomic
136 profiles) are reliable tools to evaluate the impacts of different types of environmental
137 contaminants (Zhou et al. 2018; Hussain et al. 2020). Egg production rate (EPR) and
138 hatching success (HS) were studied here as sub-lethal endpoints under different NiO
139 NPs concentrations. The response of the antioxidant defense system to NiO NPs was
140 investigated by determining the levels of some antioxidant enzymes such as catalase
141 (CAT), superoxide dismutase (SOD), and glutathione S-transferase (GST), and the
142 level of acetylcholinesterase (AChE) as a neurotoxicity indicator of oxidative damage.
143 This study is the first to focus on marine planktonic copepods, with the aim of
144 providing new knowledge to clarify the mechanisms behind NiO NPs toxicity in this
145 species.

146 Materials and methods

147 Copepod sampling and algal cultures

148 Copepods were captured in the Bizerte channel, north of Tunisia (37°16'1"N;
149 9°52'50"E), using a WP2 plankton net (200 µm mesh size) drawn by vertical tows.
150 Sampling was carried out during summer and autumn 2017. Copepods were placed in
151 cold boxes and immediately transported to the laboratory. Identification of *C.*
152 *ponticus* was performed based on their morphological and swimming characteristics,
153 using a Leica MZ125 stereomicroscope (Soler et al. 1988). Prior to exposure to
154 nanoparticles, adult females of *C. ponticus* were acclimated in seawater at 23 °C and

37 psu for 24 h, and fed the marine microalga *Isochrysis galbana*. Culture of this algal species was performed in filtered seawater (0.2 µm) with f/2 enriched media at 20 °C and on a 12-:12-h light: dark cycle. All glassware was soaked in 10% nitric acid (NHO_3) for 24 h and autoclaved at 121 °C for 15 min before use.

Nickel nanopowder preparation

Nickel oxide nanoparticles (product reference 637130) were purchased from Aldrich Sigma Chemicals. A test solution was freshly prepared before the beginning of the experiment and then diluted in filtered seawater. The NiO NPs were dispersed in ultra-pure water to obtain a concentration of 100 mg/L and the suspension was then sonicated for 20 min at 4 °C (40 KHz, 100W). The particle size of the NiO NPs was ≤50 nm with purity >99.8% and the particle morphology was cubic, according to the supplier (https://www.sigmaaldrich.com/catalog/product/aldrich/637130?lang=en&region=TN). The size of these nanoparticles (before its dispersion in ultrapure water or biological test media (powder form) was confirmed (< 50 nm) by Sousa et al. (2018a) using transmission electron microscopy. Right after dispersion in deionized water, Sousa et al. (2018a) reported a main hydrodynamic size of 342 nm which exceeds 1000 nm after 24 h and displays a negative zeta potential value.

Previous physical characterizations of other manufactured NiO NPs, different from the one we used, in seawater showed that these NPs display negative zeta potential value, non-uniform morphology and a size > 100 nm revealing their tendency to aggregate in seawater (Gong et al. 2011; Ates et al. 2016; Gong et al. 2019).

Toxicity test procedures

Acute lethal toxicity test

To determine the lethal toxicity (median lethal concentration, LC_{50}) of NiO NPs, 48-h acute toxicity tests with adult females of *C. ponticus* were performed according to ISO 14669:1999 revised 2015 and Zhou et al. (2016), with some modifications. All glass beakers were washed with 10% NHO_3 and rinsed three times with deionized water. For each NiO NP concentration tested, triplicate groups of 10 adult females were placed in 150 mL glass beakers containing 100 mL of the test solution. Five NiO NP concentrations were tested: 0, 0.5, 1, 5, 10, and 50 mg/L. Photoperiod was 12-:12-

h light: dark cycle, salinity was 37 psu, and temperature was fixed at 23 °C. During the experiment, the copepods were not fed. Mortality was recorded after 24 h and 48 h of exposure. A copepod was considered dead if no swimming or appendage movements were observed for 10 seconds.

Acute sub-lethal toxicity tests

Once the LC_{50} (48 h) for NiO NPs had been determined, acute sub-lethal toxicity tests were performed using NiO NP concentrations below the LC_{50} value. Adult females of *C. ponticus* were exposed to four sub-lethal concentrations: 50%, 5%, 0.5%, and 0.05% of the 48 h LC_{50} to study NiO NPs effect on reproduction and oxidative stress.

Reproduction

Reproductive toxicity tests were conducted on adult females according to Zhou et al. (2016) with few modifications, and then EPR and HS were monitored over 48 h of NiO NPs exposure. Three replicates were prepared for each concentration. Each replicate comprised 3-4 ovigerous females of *C. ponticus* incubated in a 150 mL glass beaker with 100 mL of filtered seawater. To prevent cannibalism, the females were separated from their eggs using a Perspex® chamber with a 200 µm mesh false bottom. The experiments were run at 37 psu and 23 °C in an incubator under a fixed 12-:12-h light:dark cycle. After 24 h, the females from each replicate were gently transferred to a new glass beaker containing fresh test solution, to measure EPR after 48 h. The number of eggs spawned was counted every 24 h under a Leica binocular microscope for each condition, and EPR was expressed as number of eggs spawned per female per day (eggs/female/day). To monitor the effect of exposure to NiO NPs on hatching success, the eggs spawned after 24 h and 48 h were then incubated for 48 h in the same solution of NiO NPs. The numbers of nauplii and unhatched eggs in each treatment were determined at the end of the incubation period.

Oxidative stress

Prior to biomarker analysis, *C. ponticus* adult females were exposed for 48 h to the same range of NiO NP concentrations as used in the reproduction tests. The experiments were conducted in triplicate for each treatment and control, following the protocol of Fossi et al. (2001). Each replicate received 150 individuals, which were placed in a 1 L glass beaker filled with filtered seawater. The temperature and salinity

were 23 °C and 37 psu, respectively. A light regime of 12-:12-h light: dark cycle was maintained. The experiment was run for 48 h and the organisms were not fed. A sample of 75 copepods was taken every 24 h from each replicate per treatment and immediately frozen at - 80 °C until measurement of biomarkers.

Biomarker analysis

Total protein

In order to determine the protein content in copepods, samples of *C. ponticus* were homogenized at 4 °C using an ultratorax in a buffer solution containing tris base (20 mM), EDTA (1 mM), dithiothreitol (1 mM), sucrose (500 mM), and KCl (150 mM) adjusted to pH 7.6 at a ratio of 1-4 (*w*/*v*) according to the protocol of Pinho et al. (2005). The homogenate was then centrifuged at 9000*g* for 30 min at 4 °C. The resulting supernatant was divided into several aliquots, which were stored at -80 °C until total protein and biomarker analysis. Total proteins were quantified by the Bradford method (Bradford 1976) using 50 µL sample protein and bovine serum albumin as the standard. The assay was performed in a microplate reader using 96 well plates. Protein concentrations were expressed in mg/L after spectrophotometric quantification at 595 nm.

Superoxide dismutase (SOD) activity measurement

Superoxide dismutase activity was measured based on the method of McCord and Foridovich (1996). In brief, 1960 µL of Na_2CO_3/$NaHCO_3$ (50 mM) (pH=10.2) were combined with 10 µL of catalase bovine, 10 µL of sample, and 20 µL of epinephrine. Total SOD activity was measured at 480 nm using a spectrophotometer, and was expressed as SOD U/mg protein.

Catalase (CAT) activity measurement

Catalase is an enzyme that catalyzes the decomposition of H_2O_2 formed by SOD in molecular water (H_2O) and molecular oxygen (O_2). Here, CAT activity was estimated according to Clairborne (1985), where the rate of disappearance of H_2O_2 is monitored by measuring the rate of decrease in H_2O_2 at 240 nm for 20 min. In brief, 50 µL of sample were added to 50 µL of 30% H_2O_2 and 950 mL of 75 mM phosphate buffer at pH=7. The results were expressed as CAT U/mg protein.

Glutathione S-transferase (GST) activity measurement

Glutathionine activity was determined by measuring the conjugation of 1-chloro-2-4-dinitrobenzene (CDNB) with GSH at 37 °C. The rate of GSH decrease is directly proportional to the level of GST activity in the sample. A total of 50 µL of sample was added to 50 µL of CDNB and 100 µL glutathione in 100 mM phosphate buffer (100 mM) at pH 7.4 (Habig et al. 1974). The results were expressed as GST nmol/min/mg protein.

Acetylcholinesterase measurement

Measurement of AChE activity was carried out according to Ellman et al. (1961), with 3 mM acetylthiocholine iodide (AcSCh) as substrate and 0.1 Mm dithiobisnitrobenzoate (DTNB) as reagent, at a controlled temperature of 20 °C. Absorbance was recorded at 410 nm for 15 min using 20 µL of sample according to Fossi et al. (2001). AChE activity was expressed in nmol/min/mg protein.

Data analysis

The results from the lethal and sub-lethal tests are presented as means ± their standard deviation. Statistical analyses of the data were performed using SPSS (version 18). The effects of the concentrations tested in each experiment were compared with those of controls using two-way ANOVA (time and treatment), followed by post-hoc Tukey's test. Data were log-transformed, if necessary, to meet the ANOVA assumption of normality and variance homogeneity. The level of significance was set at $p<0.05$. The median lethal concentration (LC_{50}) was calculated by probit analysis using SPSS.

Results

Acute lethal toxicity test and aggregation behavior

There was no mortality in the controls during the whole incubation period. The results of two-way ANOVA indicated a strong influence of both time and concentration on survival rate ($p<0.05$) (Table 1). They also showed a significant interaction between exposure time and NiO NP concentrations ($p<0.05$). The survival rate at concentrations ranging from 0.5 to 1 mg/L of NiO NPs was ≥ 90 % over the exposure

period. After 24 h, the survival rate was > 90% at a concentration of 5 mg/L and <50 % at a concentration ≥10 mg/L relative to the control groups (p<0.05). After 48 h of incubation, NiO NPs at concentrations greater than 1 mg/L induced a significant (p<0.05) reduction in survival rate, which decreased from 90±1.7 % at an NiO NPs concentration of 1 mg/L to 0 % at 50 mg/L. The calculated LC_{50} value for 24 h and 48 h of exposure was 13.83 ± 2.3 mg/L and 4.07 ± 0.5 mg/L, respectively (Table 2).

During the acute toxicity tests, black aggregates visible to the naked eye appeared at the bottom of the beaker at NiO NPs concentrations ranging from 2 to 50 mg/L after 48 h of incubation. Microscope observations revealed similar aggregates of NiO NPs on the exoskeleton of *C. ponticus* (Fig. 1a, b).

Effect on reproductive success

The effects of NiO NPs on reproductive success in *C. ponticus* were assessed by monitoring EPR over 48 h and then determining HS (Fig. 2). For both parameters, no significant (p>0.05) effect was observed at the lowest concentration of NiO NPs (0.002 mg/L) over 48 h.

A significant (p<0.05) influence of time exposure on EPR was observed for all NiO NPs treatments (Table 1). However, no significant (p>0.05) interaction was observed between time exposure and NiO NPs concentration. For the control groups, EPR varied between 11.3±0.6 eggs female^{-1} day^{-1} at 24 h and 8.3±1.1 eggs/female/day at 48 h. After 24 h, a significant reduction in EPR (p<0.05) was observed, to 7.5±1.5 eggs/female/day at 0.02 mg/L and 3.3±0.3 eggs/female/day at 2 mg/L of NiO NPs (Fig. 2a). After 48 h, EPR showed a continuous decrease, reaching 1.5±0.2 eggs/female/day at the highest concentration (2 mg/L).

There was no significant effect of time exposure (p>0.05) on HS for the different concentrations tested (Table 1). However, HS for eggs spawned after 24 h and 48 h decreased significantly (p<0.05) with increasing NiO NPs concentrations ≥0.02 mg/L. For the control groups, HS varied between 85.0±2.1% for eggs spawned after 24 h and 81.3±4.0% for eggs spawned after 48 h. The lowest values of HS, observed at the highest NiO NPs concentration tested (2 mg/L), were 24±7 % and 16±2 % for eggs spawned after 24 h and 48 h, respectively (Fig. 2 b). For this endpoint measurement, no significant interaction was observed between time exposure and NiO NPs concentration (Table 1).

Effect of NiO NPs on oxidative stress

In order to assess the mechanisms behind NiO NPs toxicity at cellular level, some oxidative enzymes (CAT, SOD, GST) and the neurotoxicity biomarker AChE were measured in *C. ponticus* after exposure to 50%, 5%, 0.5%, and 0.05% of LC_{50} (48 h) (Fig. 3). The activity of all enzymes analyzed was significantly ($p<0.05$) impacted by time exposure and NiO NPs concentrations (Table 1). In addition, a significant interactive effect was observed for exposure time and NiO NPs concentration ($p<0.05$) (Table 1).

SOD activity

The level of SOD activity in *C. ponticus* after NiO NP exposure showed significant stimulation ($p<0.05$) with increasing exposure time and NiO NP concentrations (Fig. 3a). The maximum SOD activity was 31±0.7 U/mg protein and was observed for the highest NiO NPs concentration (2 mg/L) after 48 h. For the control groups, SOD activity was 4.0±0.3 U/mg protein.

CAT activity

After 24 h of incubation, a significant ($p<0.05$) difference was observed between the control groups and copepods exposed to NiO NPs concentrations (Fig. 3b). The level of CAT activity was 71.3±2.5 U/mg protein at the highest concentration (2 mg/L), compared with 31.4±0.8 U/mg protein in control groups. After 48 h, CAT activity decreased significantly ($p<0.05$) at 0.2 mg/L (19.2±1.7 U/mg protein) and 2 mg/L (12.8±3.0 U/mg protein) compared with the control groups (32.3±2.5 U/mg protein).

GST activity

After 24 h, exposure to NiO NPs led to a significant ($p<0.05$) increase in GST activity in a dose-dependent manner (Fig. 3c). At 2 mg/L, GST activity reached 65.8±5.2 nmol/min/mg protein, in comparison with 20.4±1.7 nmol/min/mg protein for the control groups. A significantly ($p<0.05$) higher level of GST activity was also observed after 48 h relative to the values measured after 24 h of incubation.

Acetylcholinesterase activity

Exposure to NiO NPs for 24 h resulted in significant ($p<0.05$) stimulation of AChE activity at all concentrations, with a minimum activity level of 67.4±4.4 nmol/min/mg

protein at 0.002 mg/L and a maximum of 296.4±6.4 nmol/min/mg protein at 2 mg/L (Fig. 3d). After 48 h at NiO NPs concentrations ranging from 0.002 to 0.2 mg/L, AChE activity was higher than the levels observed after 24 h. However, at the highest NiO NPs concentration tested (2 mg/L), AChE activity was significantly reduced (34.1±3.3 nmol/min/mg protein) in comparison with the control group (52.37.3±2.7 nmol/min/mg protein) (p<0.05).

Discussion

Acute toxicity test and aggregation behavior of NiO NPs

The results demonstrated the lethal acute toxicity of NiO NPs on adult copepod *C. ponticus* under short-term exposure (up to 48 h), with an LC_{50} value of 13.83 mg/L and 4.07 mg/L for 24 and 48 h, respectively. To our knowledge, this is the first study to report on the lethal effect NiO NPs on marine planktonic copepods. Available data regarding the lethal effect of NiO NPs on marine organisms in general are scarce and the majority of previous research has focused on freshwater species (Table 3). To our knowledge, only one previous study has investigated the mortality effect of NiO NPs on the marine amphipod *Leptocheirus plumulosus* (Hanna et al. 2013). The results from this study indicated that NiO NPs, tested at concentrations from 500 to 2000 µg/g dry weight, provoked no mortality in the amphipod over 10 days (Hanna et al. 2013).

In freshwater crustacean species including *Daphnia magna*, Gong et al. (2016) observed a nine-fold higher value of LC_{50} (48 h) (36.79 mg/L for neonates) than found here for *C. ponticus* (Table 3). However, similar values of LC_{50} (24 h) as the 48 h LC_{50} found in the present study for *C. ponticus* were reported by Nogueira et al. (2015) for the same life stage of *D. magna* exposed to NiO NPs (Table 3). Very high LC_{50} values for NiO NPs have been recorded in other biological models at higher trophic levels, such as in adult zebrafish under acute (LC_{50} (48 h) = 760 mg/L) and chronic exposure (LC_{50} (30 days) = 45 mg/L) (Kovrižnych et al. 2013, 2014). Other studies on the toxic effects of NiO NPs have shown that exposure of the freshwater alga *Chlorella vulgaris* to a concentration of 13.7 mg/L, *i.e.*, close to our LC_{50} (24 h), reduces the viability of exposed cells to 50% after 72 h (Oukarroum et al. 2017). Kanold et al. (2016) did not observe mortality effects of nickel nanoparticles (Ni NPs)

on the early life stage of the sea urchin *Paracentrotus lividus* exposed to concentrations up to 3 mg/L of Ni NPs after 48 h, which correspond to 88 µg/L of Ni^{2+}.

In this study, NiO NPs displayed aggregation in seawater during the acute lethal and sublethal tests at concentrations ranging from 2 to 50 mg/L, after 48 h. This in line with findings observed by Sousa et al. (2018b) and Zhou et al. (2016), who observed similar behavior for NiO NPs and Ni NPs at concentrations ranging from 4 to 10 mg/L after 72 h. Like other nanoparticles (titanium oxide (TiO2) NPs, silver (Ag) NPs), NiO NPs tend to aggregate independently of the aqueous medium (freshwater, seawater, deionized water, etc.), forming larger aggregates in seawater than in freshwater due to seawater characteristics favoring aggregation (Ates et al. 2016). The agglomerates increased in size with time varying from few hundred nanometers to several microns both in seawater or freshwater (Wang et al. 2014; Ates et al. 2016; Sousa et al. 2018a). Additionally, NiO NP aggregation does not exclude the possibility of release of free dissolved Nickel ions (Ni^{2+}) in the aqueous medium test, which may also impact the physiology of the copepod (Gong et al. 2011; Sousa et al. 2018b). Unfortunately, NiO NPs dissolution behavior was not examined here to get a clear idea of the amount of dissolved Ni^{2+} released into seawater test medium that can act in NiO NPS toxicity. Previous studies indicated a low solubility of these NPs in an aqueous medium test which is higher in freshwater (7%) than in seawater (0.14 %) under short-term periods (Ates et al. 2016; Oukarroum et al. 2017; Gong et al. 2011, 2019). The availability of Ni^{2+} released into the seawater from NPs increased with time and NiO NPs concentrations (Hanna et al. 2013; Zhou et al. 2016; Oukarroum et al. 2017; Gong et al. 2011, 2019). Hanna et al. (2013) reported a slow dissolution of NiO NPs under a long-term period, where NPs dissolved over several weeks in seawater. The same authors reported a solubility of 21 % of NiO NPs after 28 days for a concentration of 10 mg/L of NiO NPs which is higher than solubility observed in others studies under short-term test (Gong et al. 2011, 2019). For 50 mg/L of NiO NPs, a concentration which causes 100 % lethality in copepods after 48 h, a previous study has reported a concentration of 110 µg/L of Ni^{2+} released in seawater after 96 h (Gong et al. 2011). Copepods are therefore exposed to both particle and dissolved forms of NiO NPs and toxicity can be linked to Ni^{2+} released ion from NPs (Sousa et al. 2018b), to the particles themselves (Capasso et al. 2014) or to the combined effect

of Ni^{2+} and NPs aggregates (Gong et al. 2011, 2019). Further work needs to be done to better explain the chemical mechanism involved in NiO NPs copepods toxicity effects and to determine the real NiO NPs concentrations.

Settling of NiO NPs, as observed on the exoskeleton of *C. ponticus* in this study, has also been observed for *Chlorella vulgaris* and the brine shrimp *Artemia salina* (Gong et al. 2011; Ates et al. 2016). Biological surface coating by NPs, is considered as the main mechanism of toxicity for no-ion releasing NPs or low soluble NPs such as NiO NPs, deemed to alter the swimming behavior of aquatic organisms which could lead to negative impacts in aquatic ecosystems (Gong et al. 2011; Noss et al. 2013; Oukarroum et al. 2017; Gong et al. 2019).

According to Sukhanova et al. (2018), the lethal toxicity effect of NiO NPs on aquatic organisms depends on the biological model (size, life stage, weight) and on nanoparticle characteristics (size, shape, behavior). To our knowledge, the LC_{50} (48 h) observed in the present study is lower than any LC_{50} value reported previously in the literature, which suggests that copepods can be a good bio-indicator of the toxic effects of NiO NPs. Comparison of our LC_{50} values with NiO NPs environmental concentrations is not possible due to the lack of data in aquatic environments. However, it is worth noting that mortality effect of NiO NPs on of *C. ponticus* was observed at concentrations that were much higher than predicted environmental concentrations, which do not exceed the µg range (Zhang et al. 2019). Thus, the concentrations reported in this study may reflect the lethal effect upon accidental exposure to NiO NPs.

Effect on reproductive traits

Our results showed that NiO NPs did not impact the reproductive performance (EPR and HS) of *C. ponticus* at a concentration of 0.002 mg/L during 48 h of exposure. However, EPR and HS were significantly affected at NiO NP concentrations ≥0.02 mg/L. Previous studies have also found that metallic nanoparticles (*e.g.*, zinc oxide (ZnO) NPs and Ni NPs) are associated with impairment of copepod reproductive traits (Zhou et al. 2016; Parlapiano et al. 2017). Time exposure and concentration had a strong influence on EPR (Table 1), which decreased with increasing NiO NPs concentration and time exposure (by 32% and 46%, respectively, at 0.02 mg/L and by 70% and 82%, respectively, at 2 mg/L after 24 h and 48 h). Similar effects have been

observed in the crustacean species *D. magna*, with NiO NPs markedly reducing offspring production at concentrations ≥0.2 mg/L after 21 days (Gong et al. 2016). Despite the difference in incubation duration and probably frequent renewal of test solution in that study, reproduction success in *C. ponticus* was impacted at much lower concentrations (≥0.02 mg/L) than those tested by Gong et al. (2016). Likewise, Zhou et al. (2016) did not observe any effect on EPR in the marine calanoid copepod *Acartia tonsa* at Ni NPs concentrations up to 0.01 mg/L over 4 days.

The results in the present study showed significant concentration-related trends in HS, which decreased with increasing NiO NPs concentrations. At the highest concentration tested here (2 mg/L), HS was reduced by 70% and 79%, respectively, for eggs produced after 24 h and 48 h. Zhou et al. (2016) also observed a negative effect of Ni NPs on HS of the copepod *A. tonsa* at a concentration of 17 mg/L, where only 9% of incubated eggs hatched. Similar responses to NiO NPs have been observed in zebrafish, including a delay in egg hatching and a reduction in HS at concentrations ranging from 100 to 800 mg/L (Kovrižnych et al. 2013). Our results provide evidence of the impact of NiO NPs on copepod reproductive success at much lower concentrations (0.02 mg/L) than those tested in previous studies on marine organisms. To our knowledge, only one study has reported similar negative effects, on reproduction of the freshwater crustacean *D. magna*, at similar NiO NPs concentrations (0.045-0.14 mg/L) to those tested in the present study (Nogueira et al. 2015). Regarding NiO NPs solubility at the sublethal concentrations tested here (0.002-2 mg/l) and according to our knowledge, there are no data in the literature on NiO NPs solubility in seawater at the same concentrations range. The study of Sousa et al. (2018b) reported a concentration of Ni^{2+} of 250 µg/L released in deionized water after 72 h from a concentration of 1.6 mg/L. Based on this finding and considering the low solubility of NiO NPs in seawater in comparison to freshwater, we can suggest that the concentration of Ni^{2+} released in seawater at concentration of 2 mg/L, under our experimental conditions, could be less or close to 250 µg/L. This concentration is well higher than the highest levels of nickel which are not expected to pose a significant risk in species in saltwater under acute (74 µg/L) and chronic (8.3 µg/L) exposure as fixed by US EPA Aquatic Life Criteria Water Quality Standards (EPA 2009).

Interestingly, the NiO NPs concentrations causing toxic effects on reproduction physiology in *C. ponticus* and *D. magna* are close to those measured in contaminated ecosystems, which are suspected to occur at µg range, suggesting that environmentally realistic concentrations of NiO NPs are likely to impact reproduction of both species. Reproduction is a crucial biological function in organisms to maintain existence of species. Impairment of reproduction in copepods by NiO NPs, as observed in this study, can result in alteration of marine community structure and in modification of the food trophic web.

Oxidative stress

In order to understand the mechanisms of NiO NPs toxicity at cellular level, changes in the activity of antioxidant enzymes and a neurotoxicity biomarker in *C. ponticus* under short-term NiO NPs exposure were investigated. The results indicated a significant modulation of the first-defense antioxidant enzymes SOD and CAT in *C. ponticus* in response to sublethal exposure to NiO NPs. The stimulation of antioxidant enzymes was possibly a defense mechanism for coping with the oxidative stress generated by NiO NPs. This is in line with previous studies showing ability of nanoparticles to generate oxidative stress in aquatic organisms under short-term exposure (*e.g.* the brine shrimp *Artemia salina*, the copepod *Calanus finmarchirus*, the fish *Nile tilapia*, etc.) (Abdel-khaled et al. 2015; Ates et al. 2016; Farkas et al. 2020).

Our results showed that antioxidant enzyme levels in *C. ponticus* were significantly influenced by time exposure and NiO NPs concentration. Expression of SOD and CAT enzymes exhibited similar patterns, with an increasing trend after 24 h that was probably linked to production of reactive oxygen species (ROS), namely O_2 and H_2O_2, in *C. ponticus* as a consequence of NiO NPs exposure, as previously observed in various freshwater microalgae and plants (*e.g.*, *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, *Lemna gibba*) (Oukarroum et al. 2015; Oukarroum et al. 2017; Sousa et al. 2018b; Gong et al. 2019). After 48 h, SOD activity showed a similar trend as at 24 h, while there was a significant reduction in CAT enzymatic activity at the two highest concentrations of NiO NPs (0.2 and 2 mg/L). This decrease might be linked to high production of H_2O_2 by SOD and limited capacity of CAT to degrade it (Ighodaro and Akinloye 2018). Such antioxidant stress response was also

reported in the marine copepods *Tigriopus japonicus* exposed to sunscreens containing Zinc oxide nanoparticle (ZnO NPs); with high ROS levels observed after 96 h at environmentally realistic concentrations of ZnO NPs (Wong et al. 2020).

In combination with the antioxidant response, exposure to NiO NPs also induced activation of the multifunctional GST enzyme over the whole exposure period (48 h). In line with our results, Farkas et al. (2020) reported that exposure to silver nanoparticles (Ag NPs) triggered an increase in gene expressions of antioxidant enzymes GST in the copepod *Calanus finmarchirus* after 96 h. GST enzyme plays a key role in biotransformation of endogenous and exogenous toxic compounds present in the cell and is also recognized as an indicator of lipid damage (Prione et al. 2016; Dasari et al. 2017). NiO NPs has previously been reported as inductor of lipids peroxidation at concentrations of 1 to 50 mg/L in *Artemia salina* and *Gracilaria lemaneiformis* (Han et al. 2012; Ates et al. 2016). Results of the present study are in accordance with the results observed for the copepod *Eucyclop sp*. exposed to titanium dioxide nanoparticle (TiO_2) combined to lead (Pb), where Glutathione peroxidase (GPx), Glutathione reductase (GR), and CAT significantly increased after contaminant accumulation confirming that the exposed copepods had suffered from oxidative stress triggered by TiO_2 and Pb (Matouke and Mustapha, 2018).

Neurotoxicity is one manifestation of oxidative stress damage induced by chemicals. The enzyme AChE hydrolyzes the neurotransmitter acetylcholine (Ach) to acetate and choline, in order to prevent constant stimulation of the synapse. The present study showed a stimulating effect on AChE activity of NiO NPs (0.002-2 mg/L) after 24 h, but we observed a significant reduction in AChE activity at 2 mg/L of NiO NPs after 48 h. Under metals exposure, AChE exhibited the same pattern in living organisms, depending on time exposure; AChE increased during the earliest hours of metal exposure and then decreased with time (Bainy et al. 2006; Emadeldeen 2014; Ensibi and Yahia, 2017).

The high level of AChE activity in *C. ponticus* observed in this study may be related to interactions between NiO NPs and acetylcholine receptors causing accumulation of free unbound acetylcholine at cholinergic receptor sites. Some authors considered the excess production of AChE activity as a compensatory mechanism related to an initial reduction in this enzyme by pollutants (Bainy et al. 2006; Ferreira et al. 2012).

Inhibition of AChE activity is commonly used as an indicator of neurotoxicity in living organisms such as copepods and cladoceran species under exposure to metal and pesticide (Forget et al. 2003; Cailleaud et al. 2007; Ensibi et Yahia. 2017). Inactivation of AChE activity at a concentration of 2 mg/L suggests that NiO NPs can affect the brain of aquatic species and cause neurotoxicity through disturbance of cholinergic neurotransmission. Similar inhibitory effects of metal nanoparticles (silicon dioxide (SiO_2), TiO_2, aluminum oxide (Al_2O_3), and aluminum (Al) NPs) on AChE have been observed previously and are reported to depend on nanoparticle concentration (Wang et al. 2009). This reduction can be explained by the excess production of AChE in *C. ponticus* ceased at a NiO NPs concentration of 2 mg/L. Previous research has suggested, not confirmed, that modulation of AChE enzyme in copepods can result in a disorder in swimming behavior, tetany, and paralysis (Forget et al. 2003; Ferreira et al. 2012). Swimming behavior response of aquatic organisms, such as fish and cladocerans (*Daphnia* sp.), under contaminant exposure, has been reported to be strongly related to AChE activity. Further, it increases as the levels of AChE enzyme increase while the opposite occurs when AChE enzyme levels decrease, impacting, therefore, the survival of living organisms (Bonansea et al. 2016; Ren and al. 2017). Therefore, alteration of swimming movement in copepods may have severe impacts in the copepods community which affect other communities of the ecosystem because it is associated with numerous vital functions like perception and acquisition of food, mating and reproduction, predator-prey interaction, respiration rate, and social behavior as stated by Mazzocchi and Paffenhofer (1999).

Changes in these biomarkers reflected the ability of NiO NPs to trigger oxidative stress and highlighted the significant contribution of time exposure and NiO NPs concentration in the response of *C. ponticus* to oxidative stress. Our results provide evidence that oxidative stress is one of the main mechanisms of nanoparticle toxicity in copepods under short-term exposure, as previously reported for other species (Sousa et al. 2018b; Gong et al. 2019).

Conclusions

This is the first study to examine the potential toxic effects of NiO NPs on a marine planktonic calanoid copepod. A LC_{50} (48 h) of around 4 mg/L was found for adult females of *C. ponticus*, a concentration that can exceed the environmental concentrations in contaminated environments. Results from sublethal tests clearly indicated that NiO NPs caused negative effects on reproductive performance in *C. ponticus* and led to oxidative stress at concentrations (0.02-2 mg/L), which are close to environmentally relevant levels. *C. ponticus* resisted to the oxidative stress generated by NiO NPs by increasing the levels of antioxidant enzymes in a concentration and time-dependent manner. These results provide a better understanding of the ecotoxicological risks of NiO NPs for marine organisms and confirm that the calanoid copepod *C. ponticus* is a suitable marine model organism for ecotoxicology studies. Future studies are required to determine the effects of NiO NPs on the entire copepod life cycle, including impacts on juvenile development and growth, and under long-term exposure.

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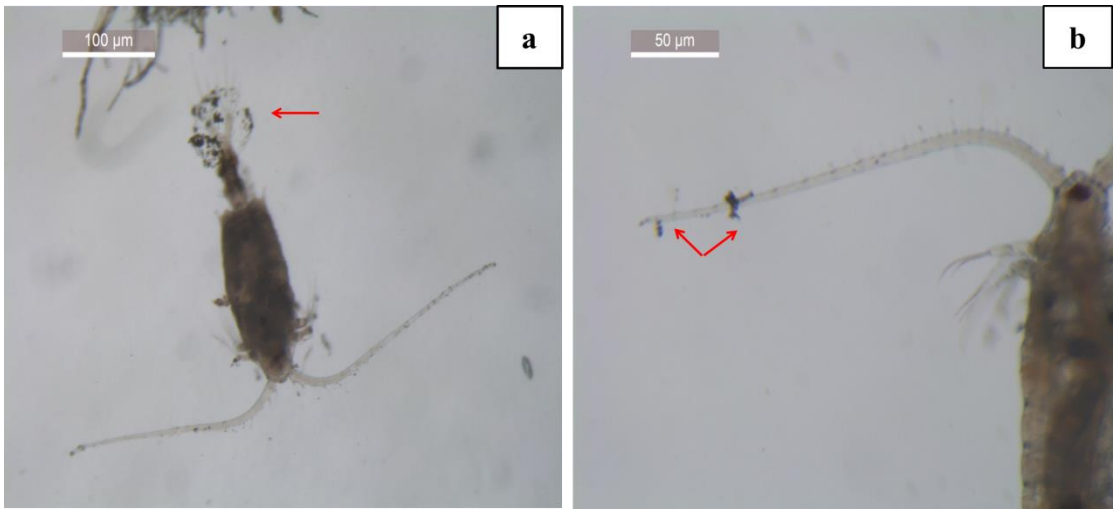
Fig. 1 Aggregates of nickel oxide nanoparticles (NiO NPs) visible by microscopy after 48 h in (**a**) on urosome and furca and (**b**) on the antennules of the marine copepod *Centropages ponticus* exposed to 2 mg/L.

Fig. 2 (**a**) Effect of nickel oxide nanoparticles (NiO NPs) on egg production rate (eggs/female/day) of *Centropages ponticus* females after 24 h and 48 h and (**b**) egg hatching success rate (%) of eggs produced by *C. ponticus* after 24 h and 48 h. Values shown are mean ± standard deviation. Letters on bars indicate significant difference between groups (Tukey's post-hoc $p < 0.05$).

Fig. 3 Effects of nickel oxide nanoparticles (NiO NPs) on activity of the enzymes (**a**) superoxide dismutase (SOD), (**b**) catalase (CAT), (**c**) glutathione S-transferase (GST), and (**d**) acetylcholinesterase (AChE) in adult *Centropages ponticus* after 24 h and 48 h. Values shown are mean ± standard deviation. Letters on bars indicate significant difference between groups (Tukey's post hoc test, $p < 0.05$).



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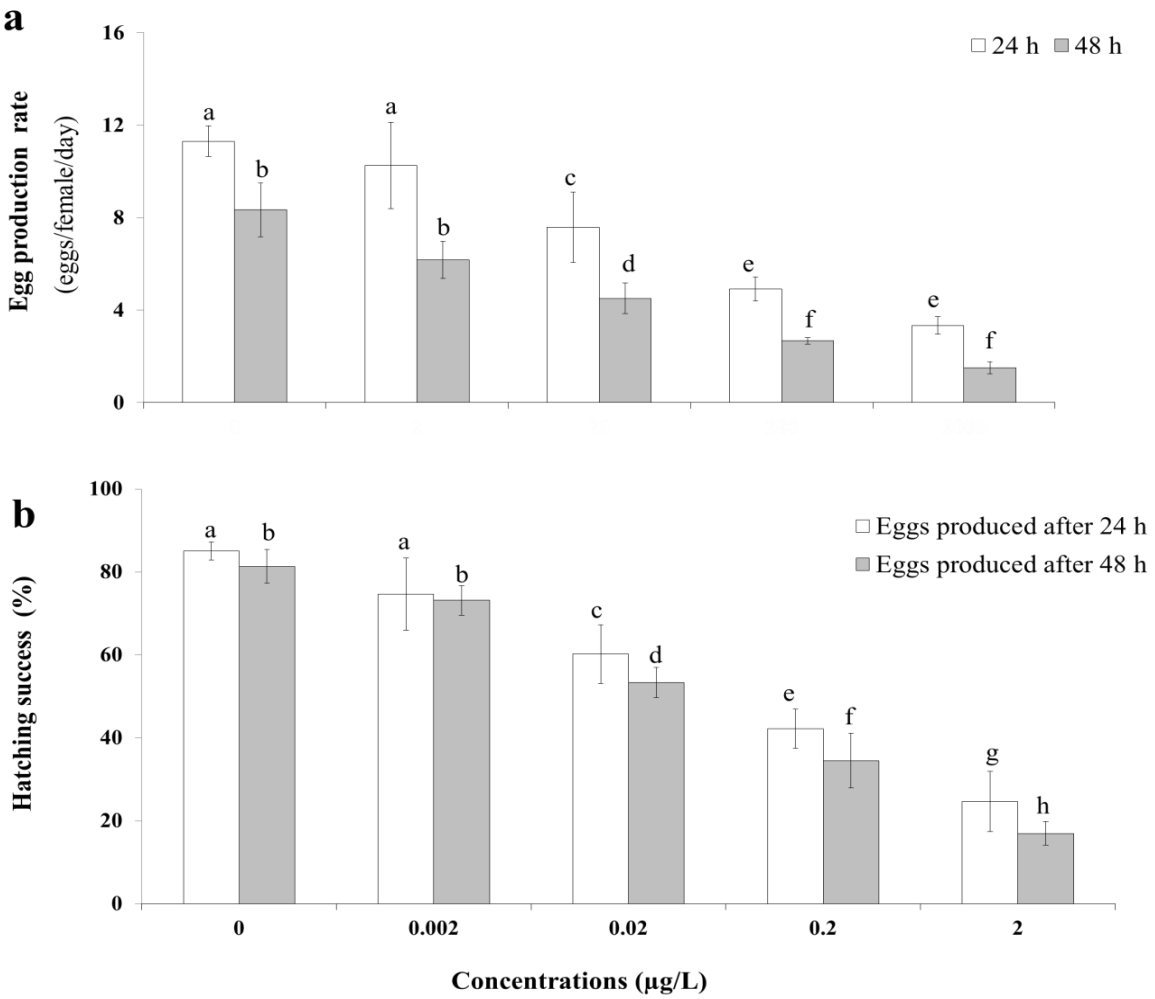

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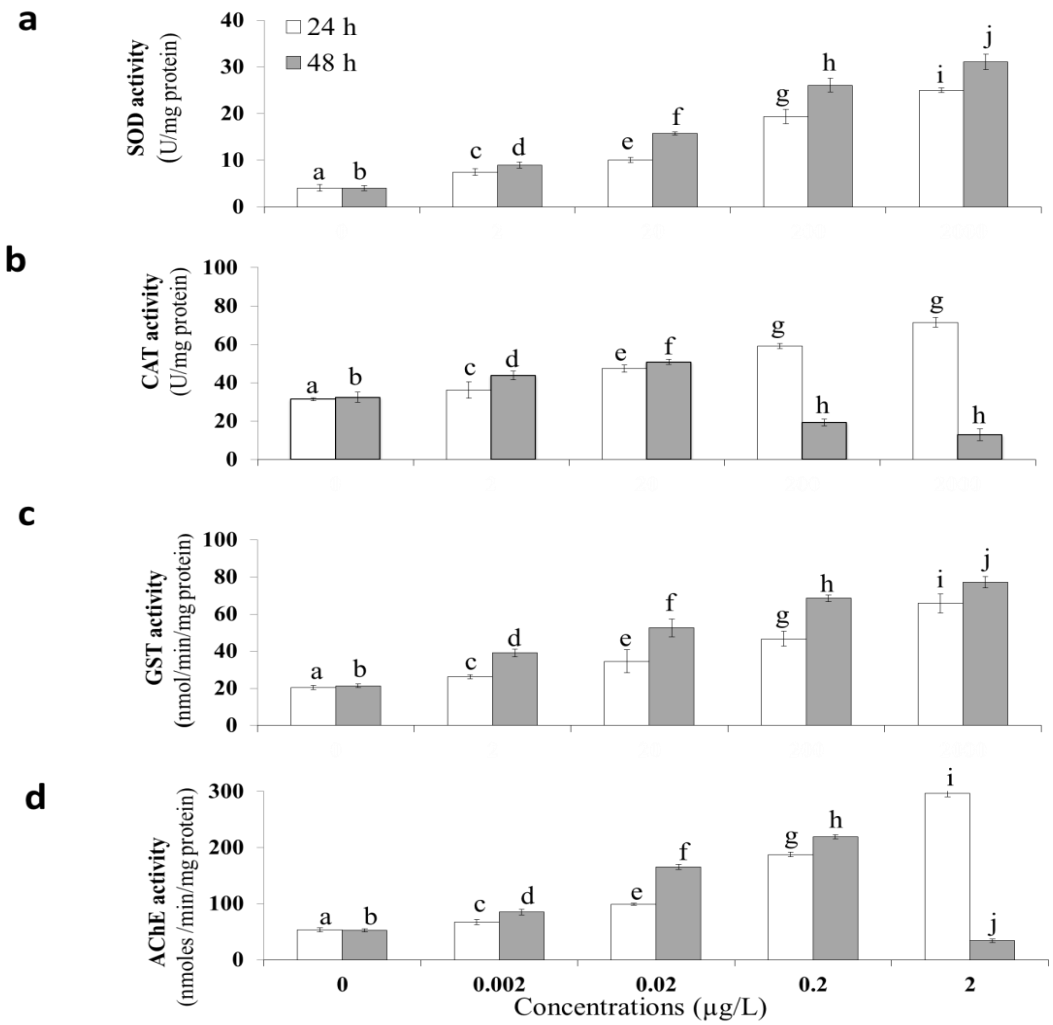

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Table 1 Synthesis of results of two-way factorial analysis of variance (ANOVA) showing the effects of concentration of nickel oxide nanoparticles (NiO NPs) and time exposure on the different parameters studied in *Centropages ponticus*: EPR = egg production rate, HS = hatching success, SOD = superoxide dismutase activity, CAT = catalase activity, GST = glutathione S-transferase activity, AChE = acetylcholinesterase.

Table 2 Lethal concentrations **(**LC_{10}, LC_{50}, LC_{70}, LC_{90}, mg/L) in *Centropages ponticus* adult females exposed to nickel oxide nanoparticles (NiO NPs) for 24 h and 48 h, with associated 95% confidence intervals (CI)**.**

Table 3 Median lethal concentration (LC_{50}) of nickel oxide nanoparticles (NiO NPs) in the marine copepod *Centropages ponticus* and in some other aquatic species.

888 **Table 1** Synthesis of results of two-way factorial analysis of variance (ANOVA)
889 showing the effects of concentration of nickel oxide nanoparticles (NiO NPs) and
890 time exposure on the different parameters studied in *Centropages ponticus*: EPR =
891 egg production rate, HS = hatching success, SOD = superoxide dismutase activity,
892 CAT = catalase activity, GST = glutathione S-transferase activity, AChE =
893 acetylcholinesterase.

Parameter	Effect of NiO NP concentration			Effect of exposure time			Concentration × Time		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Survival	23.93	4	**<0.005**	11.76	1	**<0.005**	3.17	4	**<0.005**
EPR	60.85	4	**<0.005**	66.23	1	**<0.005**	1.22	4	0.333
HS	130.43	4	**<0.005**	7.61	1	0.120	0.38	4	0.818
SOD	454.93	4	**<0.005**	105.20	1	**<0.005**	11.12	4	**<0.005**
CAT	40.49	4	**<0.005**	401.38	1	**<0.005**	240.47	4	**<0.005**
GST	191.57	4	**<0.005**	101.50	1	**<0.005**	7.50	4	**<0.005**
AChE	454.93	4	**<0.005**	105.20	1	**<0.005**	11.12	4	**<0.005**

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Exposure duration	LC_{10} (95% CI)	LC_{50} (95%)	LC_{70} (95% CI)	LC_{90} (95% CI)
24 h	4.29 (2.64-6.96)	13.83 (8.52-22.44)	22.33 (13.762-36.2)	44.6 (27.59-72.33)
48 h	1.13 (0.69-1.84)	4.07 (2.49-6.63)	6.86 (4.21-11.29)	41.45 (25.43-67.59)

925 **Table 3** Median lethal concentration (LC_{50}) of nickel oxide nanoparticles (NiO NPs)
926 in the marine copepod *Centropages ponticus* and in some other aquatic species.
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Species	Freshwater / marine species	Endpoint Test	LC_{50}	References
Chlorella vulgaris (microalga)	Freshwater species	96 h EC50 (NiO NPs 30 nm)	13.7 mg/L	Oukarroum et al. (2017)
Danio rerio (zebrafish) adults		48 h LC_{50} (NiO NPs <50 nm) 96 h LC_{50} (NiO NPs <50 nm)	760 mg/L 420 mg/L	Kovrižnych et al. (2013)
Danio rerio adults		30 day LC_{50} (NiO NPs <50 nm)	45 mg/L	Kovrižnych et al. (2014)
Danio rerio eggs		96 h LC_{50} (NiO NPs <50 nm)	1300 mg/L	Kovrižnych et al. (2013)
Daphnia magna (cladoceran) neonates (<24 h)		Immobilization (NiO NPs <50 nm) 48 h LC_{50}	36.79 mg/L	Gong et al. (2016)
		Immobilization 24 h LC_{50} (NiO NPs 100 nm) 48 h LC_{50} (NiO 100 nm) 24 h LC_{50} (NiO NPs 10-20 nm) 48 h LC_{50} (NiO NPs 10-20 nm)	14.6 mg/L 9.74 mg/L 13.98 mg/L 9.76 mg/L	Nogueira et al. (2015)
Centropages ponticus (copepod) adults	Marine species	24 h LC_{50} (NiO NPs <50 nm) 48 h LC50	13.85 mg/L 4.07 mg/L	Present study
Leptocheirus plumulosus (amphipod)		10 day LC_{50} (NiO NPs13 nm)	No mortality at ≥2000 µg/g	Hanna et al. (2013)

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