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

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36 **Abstract**

37 Excessive use of nickel oxide nanoparticles (NiO NPs) in various industrial and  
38 commercial products can lead to various negative effects in human and environmental  
39 health due to their possible discharge into the environment. Nevertheless, information  
40 about their eco-toxicological effects on marine organisms are lacking. Copepods are  
41 good eco-toxicological models because of their high sensitivity to environmental  
42 stress and their key role in the marine food webs. In this study, 48 h acute tests were  
43 conducted on the marine planktonic copepod *Centropages ponticus* to assess lethal  
44 and sublethal toxicities of NiO NPs. The results revealed LC<sub>50</sub> (48 h) of 4 mg/L for  
45 adult females. Aggregation and settling of NiO NPs were observed at concentrations  
46  $\geq 2$  mg/L. Exposure to sublethal concentrations ( $\geq 0.02$  mg/L for 48 h) had significant  
47 negative effects on reproductive success in *C. ponticus*. Egg production after 24 h and  
48 48 h decreased by 32% and 46%, respectively at 0.02 mg/L and 70% and 82%,  
49 respectively, at 2 mg/L. Hatching success was reduced by 70% and 79% at 2 mg/L for  
50 eggs produced after 24 h and 48 h respectively. Antioxidant enzymatic activity  
51 increased significantly with NiO NP concentration and time, indicating that NiO NPs  
52 can cause oxidative stress in *C. ponticus* even under short-term exposure, while  
53 significant inhibition of acetylcholinesterase activity at 2 mg/L after 48 h suggests  
54 neurotoxic effects of NiO NPs.

55 **Keywords** Metal oxide nanoparticle, Copepods, Acute toxicity test, egg production,  
56 egg hatching success, antioxidant enzymes.

## 57 **Introduction**

58 Nanoparticles (NPs) represent a serious environmental threat due to their toxicity and  
59 persistence (Geissen et al. 2015). According to the British Standards Institution, these  
60 compounds are defined as discrete pieces of material with all three external  
61 dimensions in the nanoscale (size <100 nm). Size, shape, chemical composition and  
62 structure of particle are main factors which determine the nanoparticle properties  
63 (optical, electrical, and magnetic properties) (Khan et al. 2019). Based on their  
64 chemical composition, NPs can be divided into various groups: *e.g.*, metallic,  
65 polymeric metallic oxide, semiconductor, and metallic oxide nanoparticles. Among  
66 the metallic oxide group, nickel oxide nanoparticles (NiO NPs) have widespread use  
67 in many industrial and commercial products (*e.g.*, solar cells, lithium-ion batteries,  
68 resistive random-access memory, and biosensors), due to their magnetic properties  
69 (Tadik et al. 2015). There are also multiple medical and biotechnological applications  
70 of NiO NPs, such as remediation of heavy metal contaminated water and  
71 enhancement of biogas production from macrophytes of wastewater treatment plants  
72 (Mahmoud et al. 2015; Salama et al. 2020). A recent study has reported an annual  
73 production of NiO NPs of 20 tons only in the United states (Gomes et al. 2019) which  
74 increases progressively the levels of NiO NPs exposure as stated by Avila-Arias et al.  
75 (2019). The excessive use of NiO NPs leads to their possible widespread distribution  
76 in terrestrial, estuarine, freshwater, and marine ecosystems through industrial and  
77 domestic wastewaters and aerial deposition (Wiesner et al. 2006). Despite the number  
78 of studies on NiO NPs, the environmental concentrations and distribution in field  
79 environmental matrices (water, sediment, and biota) have not yet been explored.  
80 Research investigating environmental nanoparticle concentrations in aquatic  
81 ecosystems has shown that these compounds are detectable in aquatic matrices at  
82 concentrations up to  $\mu\text{g/L}$  (Zhang et al. 2019). Overall, the behavior of nanoparticles  
83 varies with the environmental matrix (water, sediment, soil, etc.). For NiO NPs,  
84 aggregation behavior in seawater has been observed due to their low solubility and  
85 high stability (Gong et al. 2016; Oukarroum et al. 2017).

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

90 al. 2016; Oukarroum et al. 2017). In addition, Ates et al. (2016) showed that NiO NP  
91 accumulation increases with exposure concentration and duration. Acute and chronic  
92 exposures to NiO NPs have been associated with mortality effects on aquatic  
93 organisms, depending on species sensitivity (Kovrižnych et al. 2014; Oukarroum et al.  
94 2017). NiO NPs can limit cell division, as observed for the green algae *Chlorella*  
95 *vulgaris*, and can also limit reproduction in zebrafish (*Danio rerio*) (Oukarroum et al.  
96 2017). Moreover, NiO NPs have been shown to be associated with cytotoxic and  
97 genotoxic effects even under acute exposure in algae, rats, and human cells (Ada et al.  
98 2010; Oukarroum et al. 2017). The toxicity of nanoparticles is influenced by their  
99 intrinsic characteristics (size, surface area, chemical composition, crystal structure,  
100 shape, etc.) and their behavior (aggregation, dissolution) (Sukhanova et al. 2018).  
101 Studies examining the toxicity mechanism of NiO NPs have found that they cause  
102 toxicity in aquatic organisms by induction of oxidative stress through generation of  
103 high levels of reactive oxygen species (*i.e.*, hydrogen peroxide: H<sub>2</sub>O<sub>2</sub>, the superoxide  
104 anion: O<sup>2-</sup>, and the hydroxyl radical: OH) and disruption of antioxidant enzymes such  
105 as glutathione (GSH), as observed in algal cells (Manke et al. 2013; Oukarroum et al.  
106 2017). Disruption of the antioxidant system can be associated with major risks, such  
107 as neurotoxicity, lipid peroxidation, and apoptosis (Ada et al. 2010; Ates et al. 2016).

108 Despite the known toxicity of NiO NPs, marine species have received little attention,  
109 as noted by Gong et al. (2019), in comparison with freshwater species (Oukarroum et  
110 al. 2017; Sousa et al. 2018a, b). Thus, ecotoxicological studies of the effects of NiO  
111 NPs on marine organisms are needed. Copepods may be interesting marine biological  
112 models for such studies, considering their essential role in marine ecosystems as main  
113 secondary producers in food webs and as vital links between phytoplankton and  
114 higher trophic levels, to which they transfer carbon and energy (Jayalakshmi and  
115 Santhanam 2019). In addition, copepods can represent a pathway for contaminant  
116 entry for higher trophic links (Lauer and Bianchini 2010; Cailleaud et al. 2011).  
117 Moreover, copepods are known as good bio-indicators of ecosystem pollution,  
118 because of their sensitivity to several environmental disturbances (Bianchi et al. 2003;  
119 Hussain et al. 2020). These organisms can develop adaptive responses to oxidative  
120 stress using a sophisticated biochemical defense mechanism (Kim et al. 2014). The  
121 marine calanoid copepod *Centropages ponticus* is a cosmopolite species and has been  
122 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

155 37 psu for 24 h, and fed the marine microalga *Isochrysis galbana*. Culture of this algal  
156 species was performed in filtered seawater (0.2 µm) with f/2 enriched media at 20 °C  
157 and on a 12-:12-h light: dark cycle. All glassware was soaked in 10% nitric acid  
158 (NHO<sub>3</sub>) for 24 h and autoclaved at 121 °C for 15 min before use.

### 159 **Nickel nanopowder preparation**

160 Nickel oxide nanoparticles (product reference 637130) were purchased from Aldrich  
161 Sigma Chemicals. A test solution was freshly prepared before the beginning of the  
162 experiment and then diluted in filtered seawater. The NiO NPs were dispersed in  
163 ultra-pure water to obtain a concentration of 100 mg/L and the suspension was then  
164 sonicated for 20 min at 4 °C (40 KHz, 100W). The particle size of the NiO NPs was  
165 ≤50 nm with purity >99.8% and the particle morphology was cubic, according to the  
166 supplier

167 (<https://www.sigmaaldrich.com/catalog/product/aldrich/637130?lang=en&region=TN>). The  
168 size of these nanoparticles (before its dispersion in ultrapure water or biological test  
169 media (powder form) was confirmed (< 50 nm) by Sousa et al. (2018a) using  
170 transmission electron microscopy. Right after dispersion in deionized water, Sousa et  
171 al. (2018a) reported a main hydrodynamic size of 342 nm which exceeds 1000 nm  
172 after 24 h and displays a negative zeta potential value.

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

### 177 **Toxicity test procedures**

#### 178 *Acute lethal toxicity test*

179 To determine the lethal toxicity (median lethal concentration, LC<sub>50</sub>) of NiO NPs, 48-h  
180 acute toxicity tests with adult females of *C. ponticus* were performed according to  
181 ISO 14669:1999 revised 2015 and Zhou et al. (2016), with some modifications. All  
182 glass beakers were washed with 10% NHO<sub>3</sub> and rinsed three times with deionized  
183 water. For each NiO NP concentration tested, triplicate groups of 10 adult females  
184 were placed in 150 mL glass beakers containing 100 mL of the test solution. Five NiO  
185 NP concentrations were tested: 0, 0.5, 1, 5, 10, and 50 mg/L. Photoperiod was 12-:12-

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

190

#### 191 *Acute sub-lethal toxicity tests*

192 Once the LC<sub>50</sub> (48 h) for NiO NPs had been determined, acute sub-lethal toxicity tests  
193 were performed using NiO NP concentrations below the LC<sub>50</sub> value. Adult females of  
194 *C. ponticus* were exposed to four sub-lethal concentrations: 50%, 5%, 0.5%, and  
195 0.05% of the 48 h LC<sub>50</sub> to study NiO NPs effect on reproduction and oxidative stress.

196

#### 197 *Reproduction*

198 Reproductive toxicity tests were conducted on adult females according to [Zhou et al.](#)  
199 [\(2016\)](#) with few modifications, and then EPR and HS were monitored over 48 h of  
200 NiO NPs exposure. Three replicates were prepared for each concentration. Each  
201 replicate comprised 3-4 ovigerous females of *C. ponticus* incubated in a 150 mL glass  
202 beaker with 100 mL of filtered seawater. To prevent cannibalism, the females were  
203 separated from their eggs using a Perspex® chamber with a 200 µm mesh false  
204 bottom. The experiments were run at 37 psu and 23 °C in an incubator under a fixed  
205 12-:12-h light:dark cycle. After 24 h, the females from each replicate were gently  
206 transferred to a new glass beaker containing fresh test solution, to measure EPR after  
207 48 h. The number of eggs spawned was counted every 24 h under a Leica binocular  
208 microscope for each condition, and EPR was expressed as number of eggs spawned  
209 per female per day (eggs/female/day). To monitor the effect of exposure to NiO NPs  
210 on hatching success, the eggs spawned after 24 h and 48 h were then incubated for 48  
211 h in the same solution of NiO NPs. The numbers of nauplii and unhatched eggs in  
212 each treatment were determined at the end of the incubation period.

213

#### 214 *Oxidative stress*

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



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

224

## 225 **Biomarker analysis**

### 226 *Total protein*

227 In order to determine the protein content in copepods, samples of *C. ponticus* were  
228 homogenized at 4 °C using an ultratorax in a buffer solution containing tris base (20  
229 mM), EDTA (1 mM), dithiothreitol (1 mM), sucrose (500 mM), and KCl (150 mM)  
230 adjusted to pH 7.6 at a ratio of 1-4 (w/v) according to the protocol of [Pinho et al.](#)  
231 [\(2005\)](#). The homogenate was then centrifuged at 9000g for 30 min at 4 °C. The  
232 resulting supernatant was divided into several aliquots, which were stored at -80 °C  
233 until total protein and biomarker analysis. Total proteins were quantified by the  
234 Bradford method ([Bradford 1976](#)) using 50 µL sample protein and bovine serum  
235 albumin as the standard. The assay was performed in a microplate reader using 96  
236 well plates. Protein concentrations were expressed in mg/L after spectrophotometric  
237 quantification at 595 nm.

### 238 *Superoxide dismutase (SOD) activity measurement*

239 Superoxide dismutase activity was measured based on the method of [McCord and](#)  
240 [Foridovich \(1996\)](#). In brief, 1960 µL of Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> (50 mM) (pH=10.2) were  
241 combined with 10 µL of catalase bovine, 10 µL of sample, and 20 µL of epinephrine.  
242 Total SOD activity was measured at 480 nm using a spectrophotometer, and was  
243 expressed as SOD U/mg protein.

### 244 *Catalase (CAT) activity measurement*

245 Catalase is an enzyme that catalyzes the decomposition of H<sub>2</sub>O<sub>2</sub> formed by SOD in  
246 molecular water (H<sub>2</sub>O) and molecular oxygen (O<sub>2</sub>). Here, CAT activity was estimated  
247 according to [Clairborne \(1985\)](#), where the rate of disappearance of H<sub>2</sub>O<sub>2</sub> is monitored  
248 by measuring the rate of decrease in H<sub>2</sub>O<sub>2</sub> at 240 nm for 20 min. In brief, 50 µL of  
249 sample were added to 50 µL of 30% H<sub>2</sub>O<sub>2</sub> and 950 mL of 75 mM phosphate buffer at  
250 pH=7. The results were expressed as CAT U/mg protein.

## 251 *Glutathione S-transferase (GST) activity measurement*

252 Glutathione activity was determined by measuring the conjugation of 1-chloro-2-4-  
253 dinitrobenzene (CDNB) with GSH at 37 °C. The rate of GSH decrease is directly  
254 proportional to the level of GST activity in the sample. A total of 50 µL of sample  
255 was added to 50 µL of CDNB and 100 µL glutathione in 100 mM phosphate buffer  
256 (100 mM) at pH 7.4 (Habig et al. 1974). The results were expressed as GST  
257 nmol/min/mg protein.

258

## 259 *Acetylcholinesterase measurement*

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

265

## 266 **Data analysis**

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

275

## 276 **Results**

### 277 **Acute lethal toxicity test and aggregation behavior**

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

283 period. After 24 h, the survival rate was > 90% at a concentration of 5 mg/L and <50  
284 % at a concentration  $\geq 10$  mg/L relative to the control groups ( $p < 0.05$ ). After 48 h of  
285 incubation, NiO NPs at concentrations greater than 1 mg/L induced a significant  
286 ( $p < 0.05$ ) reduction in survival rate, which decreased from  $90 \pm 1.7$  % at an NiO NPs  
287 concentration of 1 mg/L to 0 % at 50 mg/L. The calculated  $LC_{50}$  value for 24 h and 48  
288 h of exposure was  $13.83 \pm 2.3$  mg/L and  $4.07 \pm 0.5$  mg/L, respectively (Table 2).  
289 During the acute toxicity tests, black aggregates visible to the naked eye appeared at  
290 the bottom of the beaker at NiO NPs concentrations ranging from 2 to 50 mg/L after  
291 48 h of incubation. Microscope observations revealed similar aggregates of NiO NPs  
292 on the exoskeleton of *C. ponticus* (Fig. 1a, b).

293

#### 294 **Effect on reproductive success**

295 The effects of NiO NPs on reproductive success in *C. ponticus* were assessed by  
296 monitoring EPR over 48 h and then determining HS (Fig. 2). For both parameters, no  
297 significant ( $p > 0.05$ ) effect was observed at the lowest concentration of NiO NPs  
298 (0.002 mg/L) over 48 h.

299 A significant ( $p < 0.05$ ) influence of time exposure on EPR was observed for all NiO  
300 NPs treatments (Table 1). However, no significant ( $p > 0.05$ ) interaction was observed  
301 between time exposure and NiO NPs concentration. For the control groups, EPR  
302 varied between  $11.3 \pm 0.6$  eggs female<sup>-1</sup> day<sup>-1</sup> at 24 h and  $8.3 \pm 1.1$  eggs/female/day at  
303 48 h. After 24 h, a significant reduction in EPR ( $p < 0.05$ ) was observed, to  $7.5 \pm 1.5$   
304 eggs/female/day at 0.02 mg/L and  $3.3 \pm 0.3$  eggs/female/day at 2 mg/L of NiO NPs  
305 (Fig. 2a). After 48 h, EPR showed a continuous decrease, reaching  $1.5 \pm 0.2$   
306 eggs/female/day at the highest concentration (2 mg/L).

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

316

317 **Effect of NiO NPs on oxidative stress**

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

325

326 *SOD activity*

327 The level of SOD activity in *C. ponticus* after NiO NP exposure showed significant  
328 stimulation ( $p<0.05$ ) with increasing exposure time and NiO NP concentrations (Fig.  
329 3a). The maximum SOD activity was  $31\pm 0.7$  U/mg protein and was observed for the  
330 highest NiO NPs concentration (2 mg/L) after 48 h. For the control groups, SOD  
331 activity was  $4.0\pm 0.3$  U/mg protein.

332 *CAT activity*

333 After 24 h of incubation, a significant ( $p<0.05$ ) difference was observed between the  
334 control groups and copepods exposed to NiO NPs concentrations (Fig. 3b). The level  
335 of CAT activity was  $71.3\pm 2.5$  U/mg protein at the highest concentration (2 mg/L),  
336 compared with  $31.4\pm 0.8$  U/mg protein in control groups. After 48 h, CAT activity  
337 decreased significantly ( $p<0.05$ ) at 0.2 mg/L ( $19.2\pm 1.7$  U/mg protein) and 2 mg/L  
338 ( $12.8\pm 3.0$  U/mg protein) compared with the control groups ( $32.3\pm 2.5$  U/mg protein).

339 *GST activity*

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

345

346 *Acetylcholinesterase activity*

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

349 protein at 0.002 mg/L and a maximum of 296.4±6.4 nmol/min/mg protein at 2 mg/L  
350 (Fig. 3d). After 48 h at NiO NPs concentrations ranging from 0.002 to 0.2 mg/L,  
351 AChE activity was higher than the levels observed after 24 h. However, at the highest  
352 NiO NPs concentration tested (2 mg/L), AChE activity was significantly reduced  
353 (34.1±3.3 nmol/min/mg protein) in comparison with the control group (52.37.3±2.7  
354 nmol/min/mg protein) ( $p<0.05$ ).

355

## 356 **Discussion**

### 357 **Acute toxicity test and aggregation behavior of NiO NPs**

358 The results demonstrated the lethal acute toxicity of NiO NPs on adult copepod *C.*  
359 *ponticus* under short-term exposure (up to 48 h), with an LC<sub>50</sub> value of 13.83 mg/L  
360 and 4.07 mg/L for 24 and 48 h, respectively. To our knowledge, this is the first study  
361 to report on the lethal effect NiO NPs on marine planktonic copepods. Available data  
362 regarding the lethal effect of NiO NPs on marine organisms in general are scarce and  
363 the majority of previous research has focused on freshwater species (Table 3). To our  
364 knowledge, only one previous study has investigated the mortality effect of NiO NPs  
365 on the marine amphipod *Leptocheirus plumulosus* (Hanna et al. 2013). The results  
366 from this study indicated that NiO NPs, tested at concentrations from 500 to 2000  
367 µg/g dry weight, provoked no mortality in the amphipod over 10 days (Hanna et al.  
368 2013).

369 In freshwater crustacean species including *Daphnia magna*, Gong et al. (2016)  
370 observed a nine-fold higher value of LC<sub>50</sub> (48 h) (36.79 mg/L for neonates) than found  
371 here for *C. ponticus* (Table 3). However, similar values of LC<sub>50</sub> (24 h) as the 48 h  
372 LC<sub>50</sub> found in the present study for *C. ponticus* were reported by Nogueira et al.  
373 (2015) for the same life stage of *D. magna* exposed to NiO NPs (Table 3). Very high  
374 LC<sub>50</sub> values for NiO NPs have been recorded in other biological models at higher  
375 trophic levels, such as in adult zebrafish under acute (LC<sub>50</sub> (48 h) = 760 mg/L) and  
376 chronic exposure (LC<sub>50</sub> (30 days) = 45 mg/L) (Kovřížnych et al. 2013, 2014). Other  
377 studies on the toxic effects of NiO NPs have shown that exposure of the freshwater  
378 alga *Chlorella vulgaris* to a concentration of 13.7 mg/L, *i.e.*, close to our LC<sub>50</sub> (24 h),  
379 reduces the viability of exposed cells to 50% after 72 h (Oukarroum et al. 2017).  
380 Kanold et al. (2016) did not observe mortality effects of nickel nanoparticles (Ni NPs)

381 on the early life stage of the sea urchin *Paracentrotus lividus* exposed to  
382 concentrations up to 3 mg/L of Ni NPs after 48 h, which correspond to 88 µg/L of  
383 Ni<sup>2+</sup>.

384 In this study, NiO NPs displayed aggregation in seawater during the acute lethal and  
385 sublethal tests at concentrations ranging from 2 to 50 mg/L, after 48 h. This in line  
386 with findings observed by Sousa et al. (2018b) and Zhou et al. (2016), who observed  
387 similar behavior for NiO NPs and Ni NPs at concentrations ranging from 4 to 10  
388 mg/L after 72 h. Like other nanoparticles (titanium oxide (TiO<sub>2</sub>) NPs, silver (Ag)  
389 NPs), NiO NPs tend to aggregate independently of the aqueous medium (freshwater,  
390 seawater, deionized water, etc.), forming larger aggregates in seawater than in  
391 freshwater due to seawater characteristics favoring aggregation (Ates et al. 2016). The  
392 agglomerates increased in size with time varying from few hundred nanometers to  
393 several microns both in seawater or freshwater (Wang et al. 2014; Ates et al. 2016;  
394 Sousa et al. 2018a). Additionally, NiO NP aggregation does not exclude the  
395 possibility of release of free dissolved Nickel ions (Ni<sup>2+</sup>) in the aqueous medium test,  
396 which may also impact the physiology of the copepod (Gong et al. 2011; Sousa et al.  
397 2018b). Unfortunately, NiO NPs dissolution behavior was not examined here to get a  
398 clear idea of the amount of dissolved Ni<sup>2+</sup> released into seawater test medium that can  
399 act in NiO NPs toxicity. Previous studies indicated a low solubility of these NPs in an  
400 aqueous medium test which is higher in freshwater (7%) than in seawater (0.14 %)   
401 under short-term periods (Ates et al. 2016; Oukarroum et al. 2017; Gong et al. 2011,  
402 2019). The availability of Ni<sup>2+</sup> released into the seawater from NPs increased with  
403 time and NiO NPs concentrations (Hanna et al. 2013; Zhou et al. 2016; Oukarroum et  
404 al. 2017; Gong et al. 2011, 2019). Hanna et al. (2013) reported a slow dissolution of  
405 NiO NPs under a long-term period, where NPs dissolved over several weeks in  
406 seawater. The same authors reported a solubility of 21 % of NiO NPs after 28 days for  
407 a concentration of 10 mg/L of NiO NPs which is higher than solubility observed in  
408 others studies under short-term test (Gong et al. 2011, 2019). For 50 mg/L of NiO  
409 NPs, a concentration which causes 100 % lethality in copepods after 48 h, a previous  
410 study has reported a concentration of 110 µg/L of Ni<sup>2+</sup> released in seawater after 96 h  
411 (Gong et al. 2011). Copepods are therefore exposed to both particle and dissolved  
412 forms of NiO NPs and toxicity can be linked to Ni<sup>2+</sup> released ion from NPs (Sousa et  
413 al. 2018b), to the particles themselves (Capasso et al. 2014) or to the combined effect

414 of Ni<sup>2+</sup> and NPs aggregates (Gong et al. 2011, 2019). Further work needs to be done  
415 to better explain the chemical mechanism involved in NiO NPs copepods toxicity  
416 effects and to determine the real NiO NPs concentrations.

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

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

#### 436 **Effect on reproductive traits**

437 Our results showed that NiO NPs did not impact the reproductive performance (EPR  
438 and HS) of *C. ponticus* at a concentration of 0.002 mg/L during 48 h of exposure.  
439 However, EPR and HS were significantly affected at NiO NP concentrations ≥0.02  
440 mg/L. Previous studies have also found that metallic nanoparticles (*e.g.*, zinc oxide  
441 (ZnO) NPs and Ni NPs) are associated with impairment of copepod reproductive traits  
442 (Zhou et al. 2016; Parlapiano et al. 2017). Time exposure and concentration had a  
443 strong influence on EPR (Table 1), which decreased with increasing NiO NPs  
444 concentration and time exposure (by 32% and 46%, respectively, at 0.02 mg/L and by  
445 70% and 82%, respectively, at 2 mg/L after 24 h and 48 h). Similar effects have been

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

453 The results in the present study showed significant concentration-related trends in HS,  
454 which decreased with increasing NiO NPs concentrations. At the highest  
455 concentration tested here (2 mg/L), HS was reduced by 70% and 79%, respectively,  
456 for eggs produced after 24 h and 48 h. Zhou et al. (2016) also observed a negative  
457 effect of Ni NPs on HS of the copepod *A. tonsa* at a concentration of 17 mg/L, where  
458 only 9% of incubated eggs hatched. Similar responses to NiO NPs have been  
459 observed in zebrafish, including a delay in egg hatching and a reduction in HS at  
460 concentrations ranging from 100 to 800 mg/L (Kovřížnych et al. 2013). Our results  
461 provide evidence of the impact of NiO NPs on copepod reproductive success at much  
462 lower concentrations (0.02 mg/L) than those tested in previous studies on marine  
463 organisms. To our knowledge, only one study has reported similar negative effects, on  
464 reproduction of the freshwater crustacean *D. magna*, at similar NiO NPs  
465 concentrations (0.045-0.14 mg/L) to those tested in the present study (Nogueira et al.  
466 2015). Regarding NiO NPs solubility at the sublethal concentrations tested here  
467 (0.002-2 mg/l) and according to our knowledge, there are no data in the literature on  
468 NiO NPs solubility in seawater at the same concentrations range. The study of Sousa  
469 et al. (2018b) reported a concentration of Ni<sup>2+</sup> of 250 µg/L released in deionized water  
470 after 72 h from a concentration of 1.6 mg/L. Based on this finding and considering the  
471 low solubility of NiO NPs in seawater in comparison to freshwater, we can suggest  
472 that the concentration of Ni<sup>2+</sup> released in seawater at concentration of 2 mg/L, under  
473 our experimental conditions, could be less or close to 250 µg/L. This concentration is  
474 well higher than the highest levels of nickel which are not expected to pose a  
475 significant risk in species in saltwater under acute (74 µg/L) and chronic (8.3 µg/L)  
476 exposure as fixed by US EPA Aquatic Life Criteria Water Quality Standards (EPA  
477 2009).



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

#### 486 **Oxidative stress**

487 In order to understand the mechanisms of NiO NPs toxicity at cellular level, changes  
488 in the activity of antioxidant enzymes and a neurotoxicity biomarker in *C. ponticus*  
489 under short-term NiO NPs exposure were investigated. The results indicated a  
490 significant modulation of the first-defense antioxidant enzymes SOD and CAT in *C.*  
491 *ponticus* in response to sublethal exposure to NiO NPs. The stimulation of antioxidant  
492 enzymes was possibly a defense mechanism for coping with the oxidative stress  
493 generated by NiO NPs. This is in line with previous studies showing ability of  
494 nanoparticles to generate oxidative stress in aquatic organisms under short-term  
495 exposure (e.g. the brine shrimp *Artemia salina*, the copepod *Calanus finmarchirus*,  
496 the fish *Nile tilapia*, etc.) (Abdel-khaled et al. 2015; Ates et al. 2016; Farkas et al.  
497 2020).

498 Our results showed that antioxidant enzyme levels in *C. ponticus* were significantly  
499 influenced by time exposure and NiO NPs concentration. Expression of SOD and  
500 CAT enzymes exhibited similar patterns, with an increasing trend after 24 h that was  
501 probably linked to production of reactive oxygen species (ROS), namely  $\text{O}_2$  and  
502  $\text{H}_2\text{O}_2$ , in *C. ponticus* as a consequence of NiO NPs exposure, as previously observed  
503 in various freshwater microalgae and plants (e.g., *Chlorella vulgaris*,  
504 *Pseudokirchneriella subcapitata*, *Lemna gibba*) (Oukarroum et al. 2015; Oukarroum  
505 et al. 2017; Sousa et al. 2018b; Gong et al. 2019). After 48 h, SOD activity showed a  
506 similar trend as at 24 h, while there was a significant reduction in CAT enzymatic  
507 activity at the two highest concentrations of NiO NPs (0.2 and 2 mg/L). This decrease  
508 might be linked to high production of  $\text{H}_2\text{O}_2$  by SOD and limited capacity of CAT to  
509 degrade it (Ighodaro and Akinloye 2018). Such antioxidant stress response was also

510 reported in the marine copepods *Tigriopus japonicus* exposed to sunscreens  
511 containing Zinc oxide nanoparticle (ZnO NPs); with high ROS levels observed after  
512 96 h at environmentally realistic concentrations of ZnO NPs (Wong et al. 2020).

513 In combination with the antioxidant response, exposure to NiO NPs also induced  
514 activation of the multifunctional GST enzyme over the whole exposure period (48 h).  
515 In line with our results, Farkas et al. (2020) reported that exposure to silver  
516 nanoparticles (Ag NPs) triggered an increase in gene expressions of antioxidant  
517 enzymes GST in the copepod *Calanus finmarchirus* after 96 h. GST enzyme plays a  
518 key role in biotransformation of endogenous and exogenous toxic compounds present  
519 in the cell and is also recognized as an indicator of lipid damage (Prione et al. 2016;  
520 Dasari et al. 2017). NiO NPs has previously been reported as inductor of lipids  
521 peroxidation at concentrations of 1 to 50 mg/L in *Artemia salina* and *Gracilaria*  
522 *lemaneiformis* (Han et al. 2012; Ates et al. 2016). Results of the present study are in  
523 accordance with the results observed for the copepod *Eucyclop sp.* exposed to  
524 titanium dioxide nanoparticle (TiO<sub>2</sub>) combined to lead (Pb), where Glutathione  
525 peroxidase (GPx), Glutathione reductase (GR), and CAT significantly increased after  
526 contaminant accumulation confirming that the exposed copepods had suffered from  
527 oxidative stress triggered by TiO<sub>2</sub> and Pb (Matouke and Mustapha, 2018).

528 Neurotoxicity is one manifestation of oxidative stress damage induced by chemicals.  
529 The enzyme AChE hydrolyzes the neurotransmitter acetylcholine (Ach) to acetate and  
530 choline, in order to prevent constant stimulation of the synapse. The present study  
531 showed a stimulating effect on AChE activity of NiO NPs (0.002-2 mg/L) after 24 h,  
532 but we observed a significant reduction in AChE activity at 2 mg/L of NiO NPs after  
533 48 h. Under metals exposure, AChE exhibited the same pattern in living organisms,  
534 depending on time exposure; AChE increased during the earliest hours of metal  
535 exposure and then decreased with time (Bainy et al. 2006; Emadeldeen 2014; Ensibi  
536 and Yahia, 2017).

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

542 Inhibition of AChE activity is commonly used as an indicator of neurotoxicity in  
543 living organisms such as copepods and cladoceran species under exposure to metal  
544 and pesticide (Forget et al. 2003; Cailleaud et al. 2007; Ensibi et Yahia. 2017).  
545 Inactivation of AChE activity at a concentration of 2 mg/L suggests that NiO NPs can  
546 affect the brain of aquatic species and cause neurotoxicity through disturbance of  
547 cholinergic neurotransmission. Similar inhibitory effects of metal nanoparticles  
548 (silicon dioxide (SiO<sub>2</sub>), TiO<sub>2</sub>, aluminum oxide (Al<sub>2</sub>O<sub>3</sub>), and aluminum (Al) NPs) on  
549 AChE have been observed previously and are reported to depend on nanoparticle  
550 concentration (Wang et al. 2009). This reduction can be explained by the excess  
551 production of AChE in *C. ponticus* ceased at a NiO NPs concentration of 2 mg/L.  
552 Previous research has suggested, not confirmed, that modulation of AChE enzyme in  
553 copepods can result in a disorder in swimming behavior, tetany, and paralysis (Forget  
554 et al. 2003; Ferreira et al. 2012). Swimming behavior response of aquatic organisms,  
555 such as fish and cladocerans (*Daphnia* sp.), under contaminant exposure, has been  
556 reported to be strongly related to AChE activity. Further, it increases as the levels of  
557 AChE enzyme increase while the opposite occurs when AChE enzyme levels  
558 decrease, impacting, therefore, the survival of living organisms (Bonansea et al. 2016;  
559 Ren and al. 2017). Therefore, alteration of swimming movement in copepods may  
560 have severe impacts in the copepods community which affect other communities of  
561 the ecosystem because it is associated with numerous vital functions like perception  
562 and acquisition of food, mating and reproduction, predator-prey interaction,  
563 respiration rate, and social behavior as stated by Mazzocchi and Paffenhofner (1999).

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

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574 **Conclusions**

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

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809 **List of figure legends**

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

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

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

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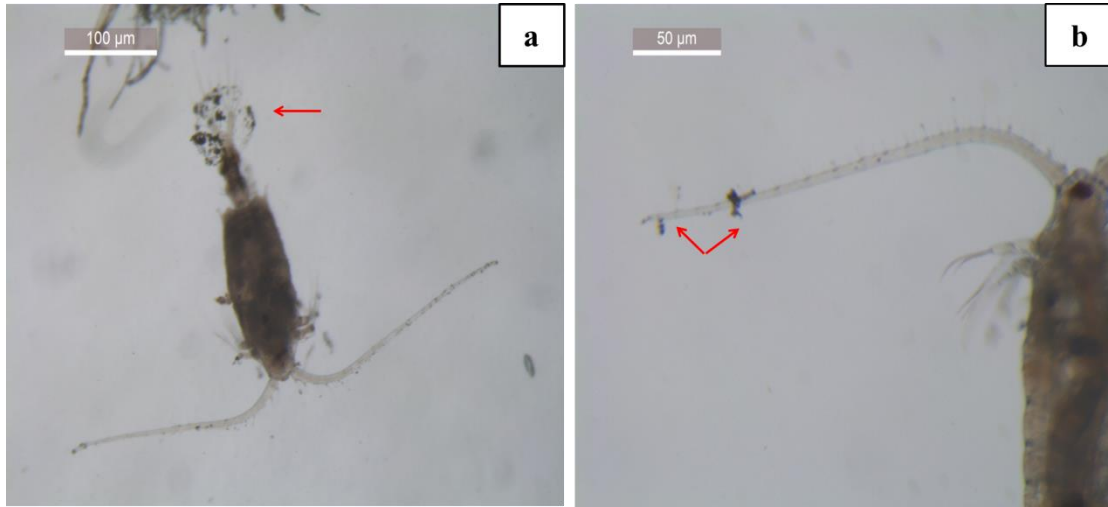
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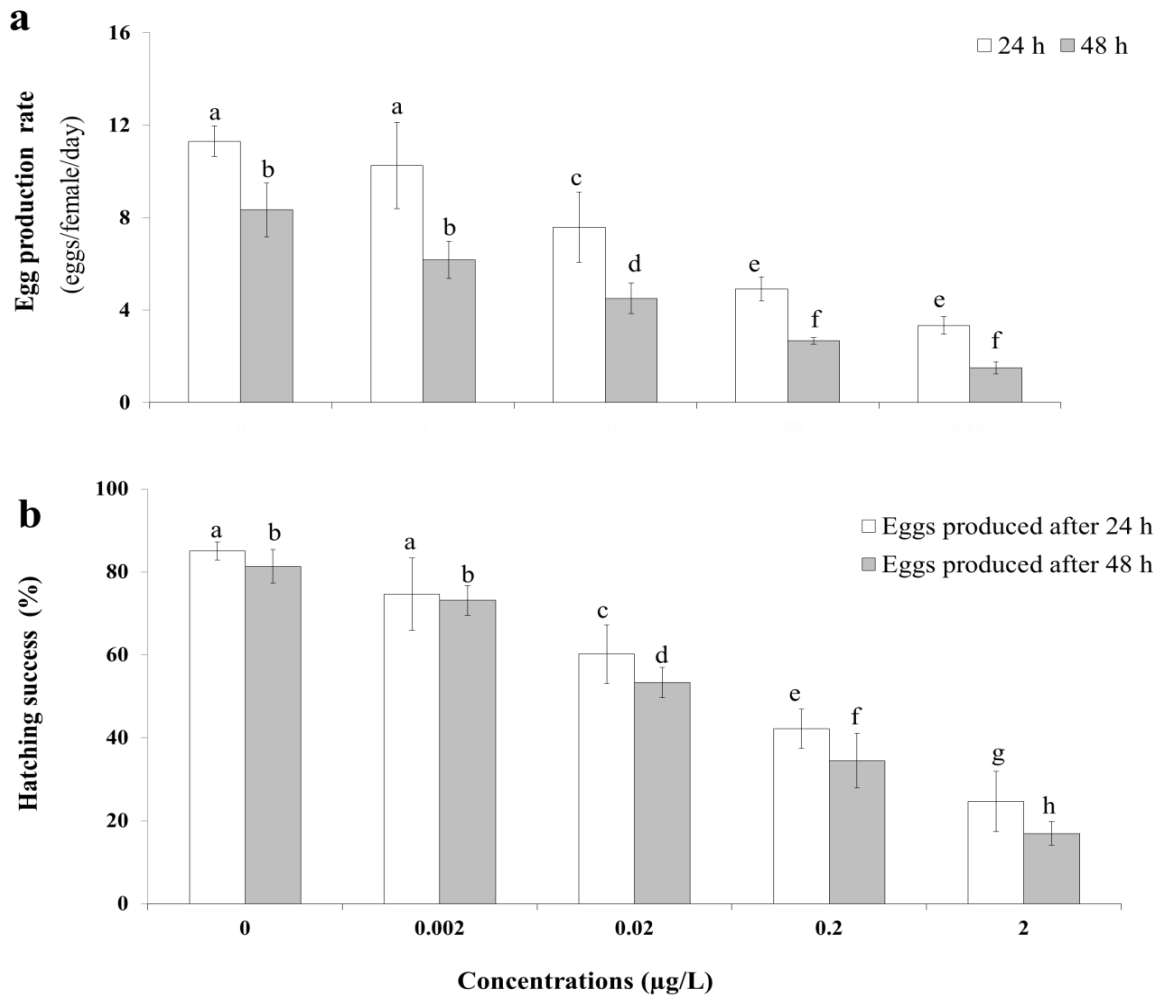
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 850 shown are mean  $\pm$  standard deviation. Different letters on bars indicate significant  
 851 difference between exposed *C. ponticus* females and controls (Tukey's post-hoc  $p$   
 852  $<0.05$ ).

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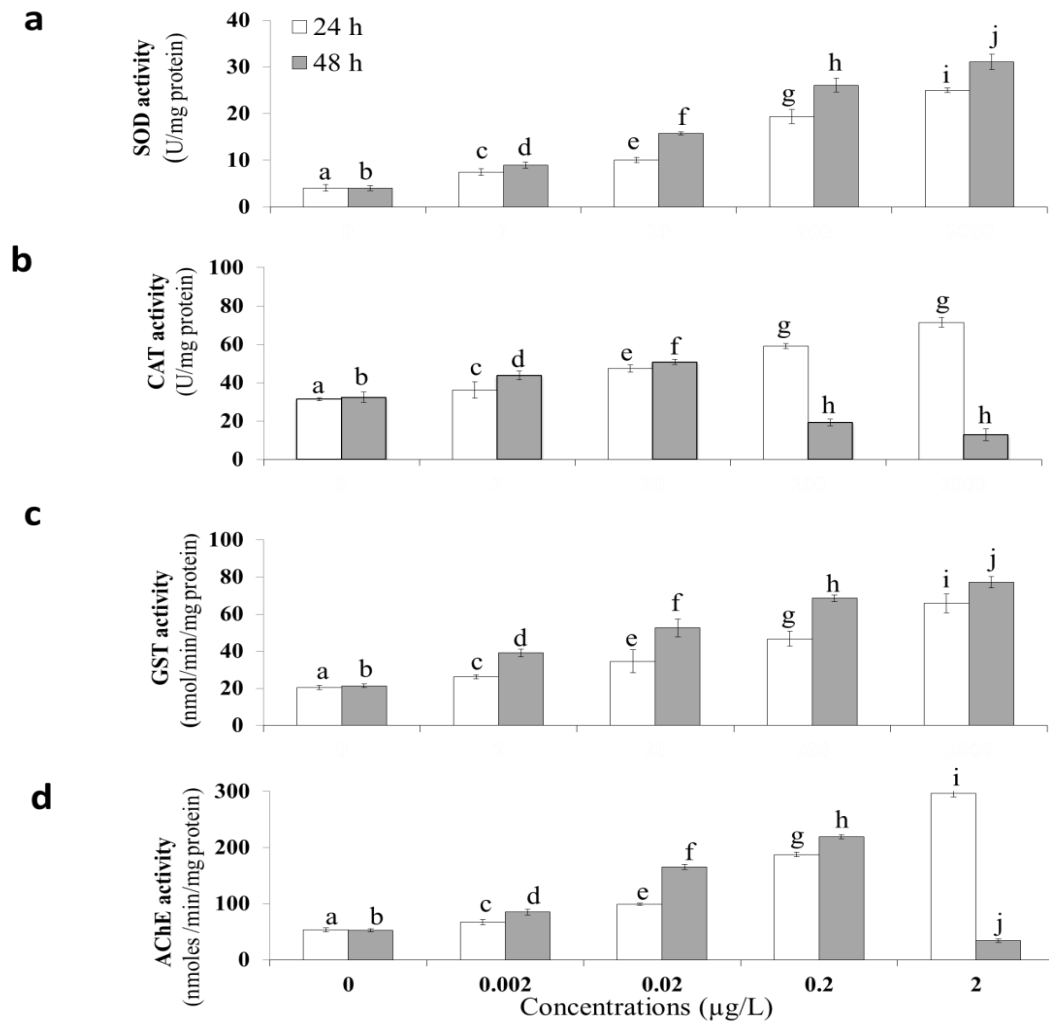
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 865 significant difference between groups (Tukey's post hoc test,  $p < 0.05$ ).

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873 **List of table legends:**

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

881 **Table 2** Lethal concentrations (LC<sub>10</sub>, LC<sub>50</sub>, LC<sub>70</sub>, LC<sub>90</sub>, mg/L) in *Centropages*  
882 *ponticus* adult females exposed to nickel oxide nanoparticles (NiO NPs) for 24 h and  
883 48 h, with associated 95% confidence intervals (CI).

884 **Table 3** Median lethal concentration (LC<sub>50</sub>) of nickel oxide nanoparticles (NiO NPs)  
885 in the marine copepod *Centropages ponticus* and in some other aquatic species.

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888 **Table 1** Synthesis of results of two-way factorial analysis of variance (ANOVA)  
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 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
<b>Survival</b>	23.93	4	<b>&lt;0.005</b>	11.76	1	<b>&lt;0.005</b>	3.17	4	<b>&lt;0.005</b>
<b>EPR</b>	60.85	4	<b>&lt;0.005</b>	66.23	1	<b>&lt;0.005</b>	1.22	4	0.333
<b>HS</b>	130.43	4	<b>&lt;0.005</b>	7.61	1	0.120	0.38	4	0.818
<b>SOD</b>	454.93	4	<b>&lt;0.005</b>	105.20	1	<b>&lt;0.005</b>	11.12	4	<b>&lt;0.005</b>
<b>CAT</b>	40.49	4	<b>&lt;0.005</b>	401.38	1	<b>&lt;0.005</b>	240.47	4	<b>&lt;0.005</b>
<b>GST</b>	191.57	4	<b>&lt;0.005</b>	101.50	1	<b>&lt;0.005</b>	7.50	4	<b>&lt;0.005</b>
<b>AChE</b>	454.93	4	<b>&lt;0.005</b>	105.20	1	<b>&lt;0.005</b>	11.12	4	<b>&lt;0.005</b>

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910 *ponticus* adult females exposed to nickel oxide nanoparticles (NiO NPs) for 24 h and  
911 48 h, with associated 95% confidence intervals (CI).

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

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925 **Table 3** Median lethal concentration (LC<sub>50</sub>) 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 <sub>50</sub>	References
<i>Chlorella vulgaris</i> (microalga)	Freshwater species	96 h EC <sub>50</sub> (NiO NPs 30 nm)	13.7 mg/L	Oukarroum et al. (2017)
<i>Danio rerio</i> (zebrafish) adults		48 h LC <sub>50</sub> (NiO NPs <50 nm) 96 h LC <sub>50</sub> (NiO NPs <50 nm)	760 mg/L 420 mg/L	Kovrižnych et al. (2013)
<i>Danio rerio</i> adults		30 day LC <sub>50</sub> (NiO NPs <50 nm)	45 mg/L	Kovrižnych et al. (2014)
<i>Danio rerio</i> eggs		96 h LC <sub>50</sub> (NiO NPs <50 nm)	1300 mg/L	Kovrižnych et al. (2013)
<i>Daphnia magna</i> (cladoceran) neonates (<24 h)		Immobilization (NiO NPs <50 nm) 48 h LC <sub>50</sub>	36.79 mg/L	Gong et al. (2016)
		Immobilization 24 h LC <sub>50</sub> (NiO NPs 100 nm) 48 h LC <sub>50</sub> (NiO 100 nm) 24 h LC <sub>50</sub> (NiO NPs 10-20 nm) 48 h LC <sub>50</sub> (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)
<i>Centropages ponticus</i> (copepod) adults	Marine species	24 h LC <sub>50</sub> (NiO NPs <50 nm) 48 h LC <sub>50</sub>	13.85 mg/L 4.07 mg/L	Present study
<i>Leptocheirus plumulosus</i> (amphipod)		10 day LC <sub>50</sub> (NiO NPs 13 nm)	No mortality at ≥2000 µg/g	Hanna et al. (2013)

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