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## A sub-individual multilevel approach for an integrative assessment of CuO nanoparticle effects on *Corbicula fluminea*

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1           **A sub-individual multilevel approach for an integrative**  
2           **assessment of CuO nanoparticle effects on *Corbicula fluminea*.**

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11 **Abstract**

12 Because they are widely used, copper oxide nanoparticles (CuO NPs) are likely to enter  
13 the aquatic environment and then reach the sediment. We have examined the effect of CuO  
14 NPs in the freshwater endobenthic bivalve *Corbicula fluminea*. Some previous studies have  
15 investigated effects at biochemical and physiological levels, but molecular endpoints are still  
16 poorly studied despite they are sensitive in early detection of NPs effect. In the present study,  
17 we have investigated short-term effects (96 h) of CuO NP (12, 30 nm; 0, 20 and 100 µg/L)  
18 using molecular endpoints as well as more conventional biochemical and physiological  
19 markers. The expression of antioxidant (CuZnSOD, MnSOD, Cat, Se-GPx, Trxr) and  
20 antitoxic (GST-Pi, HSP70, MT, Pgp, MRP1) related genes was measured at the mRNA level  
21 while antioxidant (SOD, TAC) and antitoxic (GST, ACP) defenses, energetic reserves and  
22 metabolism (ETS, Tri, LDH), and cellular damages (LPO) were assessed using a biochemical  
23 approach. The filtration rate measured at 96 hours provided information at the physiological  
24 scale. Gene expression and filtration rate were responsive to CuO NPs but the effects differed  
25 according to the NP size. The results suggest that defense mechanisms may have been set up  
26 following 30nm-NP exposure. The response to 12nm-NP was lower but still showed that  
27 exposure to 12nm-NP led to activation of cellular elimination mechanisms. The lowering of  
28 the filtration rate may have protected the organisms from the contamination. However, this  
29 raised the question of further repercussions on organism biology. Together, the results (i)  
30 indicate that CuO NP may exert effects at different levels even after a short-term exposure  
31 and (ii) point out the precocity of molecular response.

32 **Keywords:** Copper oxide nanoparticles (CuO NP), bivalve, gene expression, biochemical  
33 effects, filtration rate

34 **Capsule:** The short-term exposure of *Corbicula fluminea* to CuO NPs (12 & 30 nm) altered  
35 gene expression of cellular defense in the digestive gland and decreased filtration rate.

## 36 **1. Introduction**

37 Technological advances allow synthesis and incorporation of nanoparticles (NPs) in  
38 numerous daily commercial products. Their tiny size considerably enhances their specific  
39 surface area and confers new properties (surface reactivity, optic, catalytic, etc). Metal and  
40 metal oxide NPs are among the most manufactured NPs employed in a wide range of  
41 applications (Vance et al., 2015). Among them, copper oxide nanoparticles (CuO NPs) are  
42 used in various applications such as antimicrobial agents, agricultural biocides, catalysts or  
43 gas sensors (Hou *et al.*, 2017, Chibber and Shanker, 2017, Keller et al., 2017). Their  
44 increasing use leads inevitably to their release in the aquatic environment while their  
45 quantification and characterization in such complex matrices are not yet fully developed.  
46 Although the predictive environmental concentration (PEC) values for CuO NPs are currently  
47 estimated to be less than few  $\mu\text{g/L}$  (Keller and Lazareva, 2014), their production continues to  
48 increase from 580 tons produced in 2014 *versus* 1600 tons estimated for 2025 (Hou et al.,  
49 2017).

50 In the framework of nanotoxicology, the assessment of NP toxicity according to their  
51 physico-chemical properties remains a work in progress. Moreover, their biocide abilities  
52 raise the question of their (eco)toxicity towards non-target organisms (Keller et al., 2017). In  
53 aquatic environments, Cu-based NPs would be accumulated in the sediments of freshwater  
54 and marine environments (Keller et al., 2017). Benthic species should be of particular concern  
55 because of their close contact with contaminants and because their burrowing activities leads  
56 to a potential remobilization of contaminants (Roberts, 2012). The widespread endobenthic  
57 freshwater bivalve *Corbicula fluminea* is a good model for such assessments due to its ability  
58 to be both filter- and pelagic-feeder, but also due to its ability to strongly bioaccumulate a  
59 large amount of contaminants, such as metals (Inza et al., 1997; Shoults-Wilson et al., 2010;  
60 Marescaux et al., 2016; Hakenkamp et al., 2001). As estimated by Garner et al. (2017) for

61 freshwater ecosystems, CuO NPs may accumulate in the aquatic environment over the long  
62 term in sufficient concentration to cause potential toxicity. To the best of our knowledge,  
63 there is only one publication assessing CuO NP (20-30 nm, uncoated) effects on a freshwater  
64 bivalve. This study was conducted on the endobenthic species *Anodonta cygnea* exposed to  
65 0.25, 2.5 and 25 µg Cu/L over 12 days and showed histopathological alterations, significant  
66 bioaccumulation in the gills, and deleterious impacts in the filtration rate (Moëzzi et al.,  
67 2018). However, in freshwater bivalves the assessment of CuO NP effects in the lowest levels  
68 of biological organization are still lacking, such as in molecular and biochemical responses.

69 The main toxic mechanism reported in nanotoxicology studies is the generation of reactive  
70 oxygen species (ROS) that can induce oxidative stress, resulting in further cyto- and geno-  
71 toxicity (Klaper et al., 2014, Vale et al., 2016). A first line against reactive oxygen species  
72 (ROS) damages is the antioxidant defense pathway (Flora, 2009, Mustacich and Powis, 2000),  
73 that was affected by CuO NP in various aquatic organisms such as algae (Melegari et al.,  
74 2013), fish (Villarreal et al., 2014), anemones (Siddiqui et al., 2015) and marine bivalves  
75 (Mouneyrac et al., 2014; Kastsumiti et al., 2018). In bivalves, the antioxidant defense  
76 activities (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)) were  
77 increased after CuO NP exposure in the marine species *Mytilus galloprovincialis* and  
78 *Scrobicularia plana* exposed to 10 µg Cu/L for 3 to 21 days along with involvement of  
79 detoxification mechanisms (metallothionein (MT), glutathione-S-transferase (GST)) (Buffet  
80 et al., 2011, 2013; Gomes et al., 2011, 2012; Ruiz et al., 2015). These defenses were not  
81 sufficient to prevent damage since lipid peroxidation (Gomes et al., 2011; 2012), apoptosis  
82 (Buffet et al., 2013) and genotoxicity (Buffet et al., 2013; Gomes et al., 2013; Ruiz et al.,  
83 2015) were observed. As few dissolution was measured in each of these studies and as  
84 discussed by Ruiz et al. (2015) regarding seawater, those effects might then result of CuO  
85 particles exposure rather than Cu<sup>2+</sup>.

86 There is currently a need to investigate chemical effects at different biological levels as  
87 mentioned by the Organization for Economic Co-operation and Development (OECD) for the  
88 development of an Adverse Outcome Pathway (AOP) approach (OECD, 2018). This approach  
89 aims to provide information of causally linked events at different biological levels of  
90 organization following the exposure to a chemical and leading to an adverse health effect,  
91 which should help for regulatory purposes. In the literature, studies relying on the impact of  
92 NP across different pathways and biological levels are underrepresented so far. In this  
93 context, the use of -omic tools such as the measurement of gene expressions, protein or  
94 metabolite activities should then be particularly informative by allowing the simultaneous  
95 monitoring of a high number of responses (Snape et al., 2004; Mussali-Galante et al., 2013).  
96 In addition, the expected precocity of answers at such levels can provide an early warning  
97 signal of contamination effect that should have further repercussions on the organism  
98 physiology, and allow unraveling the pathways of toxicity mechanisms (Bigot et al., 2011;  
99 Chen et al., 2015).

100 The interactions of a contaminant with the different cellular components implicated from the  
101 cellular entry to organism response has to be taken into account. Indeed, the cellular uptake of  
102 metallic NP will occur *via* active transport (endocytosis) or passive diffusion through the cell  
103 membrane (Beddoes et al., 2015). P-glycoprotein (PGP) and multidrug resistance-associated  
104 protein 1 (MRP1), as part of the multi-xenobiotic-resistance (MXR) system, can act as  
105 detoxifier due to their role of multidrug transporter (Achard et al., 2004). If they manage to  
106 enter the cell, NPs can interact with cell targets, the first of them being proteins. In order to  
107 maintain the integrity of proteins, members of the Heat Shock Protein family may be solicited  
108 (Fabbri et al., 2008). As already mentioned, the induction of oxidative stress is expected in the  
109 presence of NP. Cells are armed with a battery of enzymes able to protect them against  
110 harmful effects of ROS. The most studied are superoxide dismutase (SOD) that allows the

111 dismutation of  $O_2^-$  to  $H_2O_2$ , Catalase (Cat) that reduces  $H_2O_2$  to water and oxygen, and  
112 glutathione peroxidase (GPx) that reduces  $H_2O_2$  or other hydroperoxides (Flora, 2009).  
113 Glutathione-S-transferase (GST) is also studied because of its contributions to antioxidant,  
114 and more generally, antitoxic mechanisms (Doyen et al., 2008). The contribution of  
115 Thioredoxin reductase (Trxr) which is mainly involved in antioxidant pathways, and its  
116 contribution in DNA synthesis, gene transcription, cell growth and apoptosis inhibition was  
117 also described (Mustacich & Powis, 2000). One major consequence of the ROS  
118 overproduction is the damage to membranes by peroxidation of their lipid components (Alves  
119 de Almeida et al., 2007). In the presence of metallic toxicants, the cell may trigger the  
120 synthesis of metallothioneins (MT). Finally, implementing such a battery of defenses against  
121 toxic effects may present an energy cost for cells. The assessment of parameters involved in  
122 the metabolic management in the cells (e.g. triglyceride content, electron transport system  
123 (ETS) activity, lactate dehydrogenase (LDH) activity) can give insights in the physiological  
124 status of the organisms and inform about the favored metabolic pathways. Studying molecular  
125 and biochemical responses in the frame of the AOP approach implies the description of these  
126 first cellular events. Experiments with short duration of exposure (24h to 96h) may provide  
127 early response ensuing the first interactions of contaminants with cells (Ankley & Edwards,  
128 2018; Lee et al., 2015). Following exposure, resident proteins trigger the response and  
129 molecular events are expected to be on first line before newly synthesized proteins can act.  
130 Therefore, working in the frame of short-term exposure duration may help in understanding  
131 the first steps of the cascade event leading to physiological impairments.

132 In bivalves, filtration has an important place in the regulation of all physiological  
133 processes because it governs the entry of water and food in organisms and thereby the entry of  
134 contaminants. It is also known that bivalves may reduce or stop the filtration process for a  
135 while when environmental conditions are stressful (Castro et al., 2018; Farris & Van Hassel,

136 2006). The filtration rate is then a good candidate for an early physiological warning  
137 (Hartmann et al., 2016).

138 The aim of this work was to provide a multiparameter and multiscale assessment  
139 (molecular, biochemical and physiological) of CuO NP effects on *C. fluminea* exposed to  
140 different concentrations (0, 20 and 100  $\mu\text{g CuO/L}$ ) in a short-term period (96 hours).  
141 Considering the importance of NP size on their stability, solubility and surface reactivity  
142 (Peng et al., 2017; Baker et al., 2014), we expected to observe different effects between the  
143 two CuO NP. A battery of gene expressions and biochemical markers involved in the  
144 previously described pathways was monitored and a filtration test was performed at the end of  
145 the exposure period. Since our aim is to depict a global modification of biological parameters  
146 associated to NP exposure, these parameters will not be analyzed individually. Instead, we  
147 developed an integrated interpretation of obtained results thanks to multivariate statistical  
148 tools.

## 149 **2. Materials and methods**

150 **All chemicals were analytical grade. Chemical and enzymes were purchased from Sigma**  
151 **(Lille, France) unless stated otherwise.**

### 152 **2.1. Tested Nanoparticles:**

153 Two CuO NPs were selected in this study. The first one was provided as CuO NP powder  
154 with a particle size of 10-100 nm specified by the manufacturer (Intrinsiq Materials Limited).  
155 These NPs were previously characterized by Buffet et al. (2011). Briefly, nanoparticles were  
156 uncapped and polyhedral with a size determined by transmission electron microscopy (TEM)  
157 of 29.5 nm in average while their hydrodynamic size in deionized water (DIW) determined by  
158 dynamic light scattering (DLS) ranged from 40 to 500 nm (194 nm average). Their mean  
159 specific surface area (SSA) was  $25.3 \text{ m}^2.\text{g}^{-1}$ . The mean zeta-potential was +26.3 mV,



160 indicative of relative stability in DIW. The suspension appeared stable for approximately 1  
161 month. A stock suspension of  $25 \text{ mg}\cdot\text{L}^{-1}$  was prepared in DIW according to Buffet et al.  
162 (2012) protocol, stored at room temperature and used during the whole experiment. The stock  
163 suspension was sonicated for 5 min (Sonorex super RK 510, 160 W) before each use and was  
164 called "30nm-NP" in this study.

165 The second tested CuO NPs, called "12nm-NP", were supplied by PlasmaChem GmbH  
166 (Germany) and suspended by the CEREGE laboratory in Milli-Q water at  $518 \text{ mg CuO/L}$ .  
167 They were previously characterized by Ortelli et al. (2017). The particles were uncoated and  
168 semi-spherical with a size reported of  $12 \pm 8 \text{ nm}$  measured by TEM. Their mean  
169 hydrodynamic size in DIW was  $140.5 \text{ nm}$  (DLS) and their SAA was  $47 \pm 1.7 \text{ m}^2\cdot\text{g}^{-1}$ . The zeta  
170 potential in ultrapure water was  $+28.1 \pm 0.6 \text{ mV}$ , indicating a relative stability in such media.  
171 This stock suspension was homogenized by magnetic stirring for a few minutes before  
172 injections (Ortelli et al., 2017).

173 **2.2. Collection and acclimatization of organisms:**

174 Freshwater Asian clams *C. fluminea* ( $3.1 \pm 0.3$  cm) were hand-collected in June 2016 in the  
175 Moselle River, La Maxe (49°09'42.9"N 6°11'55.0"E, France) at a water temperature of 21°C.  
176 The physico-chemical characteristics of the site water were previously measured (NH<sub>4</sub>: 0.03  
177 mg N/L; NO<sub>2</sub>: < 0.05 mg N/L; NO<sub>3</sub>: 1.69 mg N/L; PO<sub>4</sub>: 0.01 mg P/L; P tot: 0.10 mg P/L; Cl<sup>-</sup>:  
178 214 mg/L; SO<sub>4</sub>: 52 mg/L; TAC: 2.3 meq; MEST: 15.4 mg/L; MVS: 37.2%; Ca<sup>2+</sup>: 117 mg/L;  
179 Mg<sup>2+</sup>: 9.3 mg/L; Na<sup>+</sup>: 61.4 mg/L; K<sup>+</sup>: 4.2 mg/L; DCO: 52 mg/L; DBO5: 4 mg/L; Tot A: 9.54  
180 meq/L; Tot C: 9.4 meq/L; Bal.: -0.7%; pH: 8.0; Cond: 1040 μS/cm; dissolve Cu: 1 μg/L; Cu  
181 tot.: 1.7 μg/L). After sampling, the clams were transported to the laboratory in their water of  
182 origin in plastic coolers. They were acclimated progressively to the experimental conditions in  
183 a temperate room (15 °C) for 3 days by gradually increasing the proportion of artificial water  
184 that will be used for the experiment. The artificial water was prepared using commercial salt  
185 (Tropic Marin<sup>®</sup>, Tropicarium Buchshlag Dreieich, Germany) diluted in DIW at 1.5 psu. The  
186 elemental composition of this salt (major cations, major anions, nutrients and trace elements)  
187 was assessed by Atkinson and Bingman (1997). The media was continuously aerated and a  
188 natural photoperiod was kept (16h light: 8h dark). One day before the experiment, two  
189 organisms per beaker were placed in 200 mL of water in order to acclimate them to the  
190 device.

191 **2.3. Experimental design**

192 *C. fluminea* were exposed to 0 (controls), 20 and 100 μg CuO/L of each NP (30nm-NP and  
193 12nm-NP) for 96 hours at 15°C in artificial water (1 *C. fluminea*/100 mL). The consideration  
194 of the SSA of each NP lead to 0.000506 m<sup>2</sup>/L and 0.00253 m<sup>2</sup>/L for 30nm-NP at 20 μg/L and  
195 100 μg CuO/L, respectively, and 0.00094 m<sup>2</sup>/L and 0.0047 m<sup>2</sup>/L for 12nm-NP at 20 μg/L and  
196 100 μg CuO/L, respectively. The water was renewed every day. No aeration and no feeding

197 were performed in order to avoid any interference with NP. The natural photoperiod (16h  
198 light: 8h dark) was kept during the entire duration of the experiment.

199 The experiment was performed in beakers containing 200mL of medium and two clams.  
200 Twelve beakers were used for each combination treatment x date (144 beakers in total, i.e.  
201 288 clams). Twelve clams per treatment were sampled before water renewal at 24 and 96  
202 hours for biochemical (6 individuals) and for molecular analyses (6 individuals). At these  
203 time steps, 5 individuals were transferred in clean water to depurate before the  
204 bioaccumulation measurements. The remaining clams in each treatment were in need of help  
205 in case of mortality, but were actually not used. After 96 hours, a filtration test was also  
206 conducted on individuals kept for the bioaccumulation measurement. The total copper  
207 concentrations were measured in the water column and in the organisms (digestive gland and  
208 remaining tissues).

#### 209 **2.4. Measurements of water and tissue copper concentrations**

210 As the media was changed daily, the water was sampled for total Cu measurement  
211 immediately after NP introduction and after 24 hours (just before the next renewal) in 3  
212 beakers per treatment and time selected randomly. This procedure was applied three times: at  
213 the start of the exposure and at the renewal at 24 and 72 hours of exposure. Five clams per  
214 treatment were dissected after 24 hours of depuration at each time step (0, 24, 96 hours) for  
215 Cu measurement. Then, the digestive gland and the remaining soft tissues were selected,  
216 freeze-dried, weighed and digested in 69% HNO<sub>3</sub> for 24 hours at 60°C. Concentrations were  
217 determined by flame for concentrations above 10µg Cu/L (Perkin-ELMER Analyst 100) or  
218 by graphite furnace for concentrations below 10 µg/L (VARIAN Spectraa 800 with ZEEMAN  
219 correction) atomic absorption spectrophotometry for each individual clam. Certified water  
220 (SRM 3114) was used to check the accuracy of the quantification for the water samples (20.2  
221 ± 1.7 µg Cu/L for 20 ± 1 µg Cu/L) and certified lobster hepatopancreas (TORT-2) to check

222 the accuracy of the quantification for tissue samples ( $97.1 \pm 2.6 \mu\text{g Cu/g}$  for  $106 \pm 10$   
223  $\mu\text{gCu/g}$ ). Metal concentration in clams are expressed as  $\mu\text{g.g}^{-1}$  dry weight (DW) tissue and  
224  $\mu\text{g.L}^{-1}$  for water media.

## 225           **2.5. Measurements of gene expression**

### 226   **2.5.1. Preparation of RNA extraction**

227   *C. fluminea* digestive glands of 6 individuals per treatment were excised, rinsed in PBS 1%,  
228   and transferred into 1 mL of RNA lysis solution (Qiagen, Hilden, Germany) kept in 4°C.  
229   Then, the tissues can be kept during a maximal duration of 1 month. After a manual removal  
230   of non-digestive tissue, digestive glands were stored in -80°C until subsequent analysis.

231   The total RNA was extracted from individual digestive gland using an RNeasy mini kit  
232   (Qiagen, Hilden, Germany) according to the manufacturer protocol. Genomic DNA was  
233   digested after extraction with DNase I and total RNA was purified with RNeasy mini kit.  
234   RNA purity and quantity were assessed by optical density measurements (OD 260 nm and  
235   OD ratio 260/280 and 260/230). RNA integrity was assessed by capillary electrophoresis  
236   using Bioanalyser 2100 (Agilent, CA, USA).

### 237   **2.5.2. RT-qPCR analysis**

238   RT-qPCR was then performed on 12 genes and the sequences used in this study are listed in  
239   Supporting data Table S1. Primers were designed using Primer3Plus and purchased from  
240   Eurofins Genomic (Ebersberg, Germany). The cDNA was synthesized in a final volume of 20  
241   μL using 1 μg of RNA, 2.5 μM of random hexamer primers and SuperScript® III reverse  
242   transcriptase according to the manufacturer's instructions (Invitrogen, CA, USA). The qPCR  
243   reaction was performed as described in Koehlé-Divo et al. (2019). Briefly, 45 ng of cDNA,  
244   water (no template control) or total RNA (45 ng/reaction; no RT control) were used as a  
245   template, from 200 to 300 nM of primers (see table S1) and Fast SYBR® green master mix  
246   (Applied Biosystem®, CA, USA ) in a reaction mixture with a final volume of 20 μL. The  
247   cycling conditions were 20 s at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C.  
248   The melting curve was used to check the specificity of the amplicons and their sizes were

249 verified on agarose gel. All PCR amplifications were performed on each biological replicate  
250 using the StepOnePlus RT-PCR system (Applied Biosystems®). Gene expression levels were  
251 analyzed using the relative quantification method ( $\Delta\Delta\text{Ct}$ ) (Livak and Schmittgen, 2001). Two  
252 genes were selected as potential normalizing genes (S3 and  $\beta$ -actin), but only  $\beta$ -actin has  
253 revealed sufficient stability in our exposure conditions.  $\beta$ -actin was then used as a reference  
254 for normalization. The control condition (0  $\mu\text{g/L}$ ) was chosen as the reference condition. The  
255 final treated/control ratio and the pooled standard deviation were calculated based on the  
256 mean  $\Delta\text{Ct}$  of the biological replicates. Statistical analyses were performed separately for each  
257 exposure duration (24 & 96 hours) as described below (§2.8).

## 258 **2.6. Biochemical biomarker analysis**

259 After removal of the shell, the *C. fluminea* digestive gland was excised, kept in ice and stored  
260 at  $-80^{\circ}\text{C}$  until analysis. Each digestive gland of six clams per treatment were separately  
261 treated as described in Sroda and Cossu-Leguille (2011) for cellular biomarker measurements.  
262 The digestive glands were defrosted, weighed and crushed in a 50 mM phosphate buffer at pH  
263 7.6 and at  $4^{\circ}\text{C}$  and supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1  
264 mM L-serine borate mixture as protease inhibitor at a 8/1 volume/weight ratio. The mixture  
265 was then centrifuged at  $250\times g$  at  $4^{\circ}\text{C}$  during 5 min and supernatant was divided in two parts:  
266 one for the assay of total protein, LPO, ACP (acid phosphatase) and ETS activities, while the  
267 other part was centrifuged during 20 min at  $1,000\times g$  at  $4^{\circ}\text{C}$  and the resulting supernatant was  
268 again centrifuged 50 min at  $20,000\times g$ . The final supernatant corresponded to the cytosolic  
269 fraction that was used for enzyme activity.

270 Biomarker analysis was then performed on the automated spectrophotometer analyser  
271 (Konelab 20 XTi, Thermo Scientific) by using protocols described in Bertrand et al. (2016).  
272 Briefly, total protein content ([prot]), triglyceride content (TRI), lipid peroxidation (LPO)),

273 acid phosphatase (ACP) and electron transport system (ETS) mitochondrial activities were  
274 measured using the whole homogenate whereas total protein content ([prot]), total antioxidant  
275 capacity (TAC), glutathione-S-transferase (GST), lactate deshydrogenase (LDH) and  
276 superoxide dismutase (SOD) activities were measured in cytosolic fractions. The SOD  
277 activity was assessed using the WST-1 method (Ukeda et al., 1997) adapted to *C. fluminea*.  
278 Statistical analyses were performed separately for each exposure duration (24 & 96 hours) as  
279 described below (§2.8).

## 280 **2.7. Filtration rate measurements**

281 Five clams per treatment were placed separately in 50 mL of neutral red solution of 5 mg.L<sup>-1</sup>  
282 in the dark at 15 °C at the end of the exposure. Clams were placed in the middle of each  
283 beaker and were allowed to filter for 2 hours. Then, clams were picked up and placed in clean  
284 artificial water to let them depurate for 24 hours. After depuration, the clams were used for Cu  
285 measurements as described in §2.4. The beakers containing the neutral red solution were  
286 acidified to pH 5.0 with HCl 37% immediately after removal of the organisms and agitated  
287 for homogenization. Then, 1 mL was sampled twice in each beaker and the absorbance was  
288 read at 530 nm. The neutral red concentration in each beaker was then calculated using the  
289 standard neutral red curve. Filtration rate (f) was calculated according to Coughlan (1969):  $f =$   
290  $(V/(n \times t)) \times \log (C_0/C_t)$  with V corresponding to the total volume of neutral red solution (100  
291 mL), n the number of individuals, t the time (h), C<sub>0</sub> and C<sub>t</sub> the initial and final neutral red  
292 concentrations in the beakers, respectively.

## 293 **2.8. Statistical analysis**

294 Statistical analyses were conducted using R (R Core Team, 2014). For the filtration rate and  
295 the bioaccumulation results, the homoscedasticity and the normality were checked by Levene  
296 and Shapiro tests, respectively. As the conditions of use were confirmed, these data were

297 analyzed using a one-way anova test in order to compare both NPs separately from their  
298 respective controls for the filtration rate results. Significant results were defined with a  
299 threshold of  $p < 0.05$ . A two-way anova was performed on the bioaccumulation dataset in  
300 order to compare each condition over time.

301 The gene expression dataset as well as the biochemical markers were analyzed using  
302 multivariate analyses. First, a manova (multivariate ANOVA) was performed to assess if  
303 significant differences could be depicted according to the tested factors. These results helped  
304 us to design each Partial Least Square Discriminant Analyses (PLS-DA) highlighting the  
305 actors of those differences. Only the variables (*i.e.* gene expressions or biochemical  
306 biomarkers) with a Variable Importance in Projection (VIP) score above 0.8 were considered.  
307 Finally, Hotelling  $T^2$  tests were performed on PLS-DA results to more thoroughly explore the  
308 differences between the exposure conditions.

### 309 **3. Results**

#### 310 **3.1 Water and tissue Cu concentrations**

311 The control beakers contained  $0.56 \pm 0.38 \mu\text{g Cu/L}$  throughout the entire duration time.  
312 Measurements of total Cu in the contaminated waters are reported in fig. 1 and are expressed  
313 as percentage of recovery compared to the nominal introduced concentrations. Cu  
314 concentrations in water immediately after the introduction of NPs were close to the intended  
315 concentrations for both  $20 \mu\text{g CuO/L}$  treatments with  $98 \pm 10 \%$  and  $92 \pm 8 \%$  recovery for  
316 30nm-NP and 12nm-NP, respectively. High recoveries were also measured for both  $100 \mu\text{g}$   
317 CuO/L treatments with 30nm-NP and 12nm-NP presenting  $82 \pm 4 \%$  and  $84 \pm 8 \%$  recovery,  
318 respectively. Cu concentrations were also measured before each renewal (after 24 hours) and  
319 showed a decrease of  $74 \pm 1 \%$  in all NP treatments ( $20 \& 100 \mu\text{g/L}$ ) after 24 hours in the test  
320 media.



321 Except for some slight variations, measurements of total Cu concentrations in the *C. fluminea*  
322 digestive gland (fig. 2) showed no significant accumulation of copper during the whole  
323 experiment duration. Additional measurements of Cu were performed on the remaining soft  
324 tissues (see fig. S1) but no significant accumulation was measured either.

### 325 **3.2 Gene expression**

326 The Manova analysis performed on the 24 hour gene expression dataset indicated a significant  
327 concentration effect ( $F= 3.5_{4-60}$  df,  $p < 0.05$ ) and a significant interaction of NP type with NP  
328 concentration ( $F= 5.4_{4-60}$  df,  $p < 0.001$ ) while no effect of NP type alone ( $F= 1.0_{2-29}$  df,  $p= 0.37$ )  
329 was recorded. The pairwise comparisons (fig. 3, Table, left side) indicated significant  
330 differences between control organisms and the ones exposed to the highest concentration (100  
331  $\mu\text{g CuO/L}$ ) for 30nm-NP while the 20  $\mu\text{g CuO/L}$  treatment for 12nm-NP was different from  
332 both control and 100  $\mu\text{g/L}$  treatments. The PLS-DA presented in fig. 3 (left side) highlighted  
333 these differences. The horizontal axis separated 30nm-NP treatments by lowered expression  
334 of genes involved in antioxidant defenses (CAT, GST-pi, Trxr, CuZnSOD, MnSOD & Se-  
335 GPx) when exposure concentration increased. Both control and 100  $\mu\text{g/L}$  treatments for  
336 12nm-NP were relatively close while 20  $\mu\text{g/L}$  treatment was different, mainly along the  
337 vertical axis, presenting more PGP and MRP1 transcripts than the two other groups.

338 The Manova analysis conducted on the 96 hour gene expression dataset indicated a significant  
339 impact of NP type alone ( $F= 17_{2-29}$  df,  $p < 0.001$ ) and of its interaction with NP concentration  
340 ( $F= 4.1_{4-60}$  df,  $p < 0.01$ ) while no significant difference was shown for the concentration effect  
341 alone ( $F= 1.1_{4-60}$  df,  $p = 0.37$ ). The pairwise comparisons (fig. 3, table right side) only showed  
342 a clear discrimination between both NP types (30nm-NP & 12nm-NP) at the highest exposure  
343 concentration (100  $\mu\text{g/L}$ ). The associated PLS-DA is presented in fig. 3 (right side). Both NP  
344 types at 100  $\mu\text{g/L}$  were separated by the vertical axis that showed a higher mRNA content of  
345 MT and GST-pi in 30nm-NP exposed organisms compared to those exposed to 12nm-NP.

346 The horizontal axis described by MRP1, PGP and Trxr expressions did not allow a clear  
347 separation between groups, but 12nm-NP exposed organisms tended to display an expression  
348 slightly more pronounced for these genes than 30nm-NP exposed ones did. This is in  
349 agreement with the results observed at 24 hours when MRP1 and PGP displayed more  
350 transcripts only in organisms exposed to the lower concentration.

### 351 **3.3. Biochemical markers**

352 The PLS-DA analyses using biochemical biomarkers measured in *C. fluminea* digestive gland  
353 after exposure to the two CuO NPs (30nm-NP & 12nm-NP) are presented in fig. 4. The  
354 Manova analysis performed on the 24 hour- and 96 hour- biochemical marker datasets did not  
355 indicate any significant effect of NP concentration (24h:  $F = 0.45_{2-29}$  df,  $p = 0.65$ ; 96h:  $F =$   
356  $0.2.9_{2-29}$  df,  $p = 0.07$ ), NP type (24h:  $F = 1.95_{4-60}$  df,  $p = 0.11$ ; 96h:  $F = 1.99_{4-60}$  df,  $p = 0.11$ ) or  
357 interaction between them (24h:  $F = 0.1.67_{4-60}$  df,  $p = 0.17$ ; 96h:  $F = 0.86_{4-60}$  df,  $p = 0.49$ ). The  
358 associated Hotelling  $T^2$  tests were in accordance with the Manova's results, showing no  
359 significant difference between all exposure groups at each time step.

### 360 **3.4. Filtration rate**

361 The filtration rates measured at the end of the exposure (96 hours) are shown in fig. 5. No  
362 significant difference was obtained between both controls ( $t = 2.88_{3,8}$  df,  $p = 0.48$ ). The  
363 organisms exposed to 30nm-NP presented a significant decrease of filtration rate when  
364 exposed to the highest concentration (100  $\mu\text{g}$  CuO/L) and the ones exposed to 12nm-NP  
365 presented a significant decrease of filtration activity for both exposure concentrations (20 &  
366 100  $\mu\text{g}$  CuO/L).

## 367 **4. Discussion**

368 The aim of this study was to define if different sizes of CuO NP may have different effects on  
369 *C. fluminea* exposed in a short-term period to concentrations of copper potentially found in

370 the environment. In freshwater ecosystems, the Cu concentrations can range from 0.04 to 294  
371  $\mu\text{g/L}$  and can even reach 20  $\text{mg/L}$  in extremely polluted areas (Tran et al., 2003 and  
372 references therein). These concentrations generally relate to dissolved and micrometric Cu,  
373 but the nanoparticulate forms could be a part of this contamination. The selected  
374 concentrations in this study were included in the Cu concentration range measured in the  
375 environment, but also in those estimated by Chio et al. (2012) for Cu NP in Taiwanese rivers  
376 with a confidence interval of 95% (10 – 920  $\mu\text{g/L}$ ), but they were more than 200 times higher  
377 than the PEC values reported more recently in water by other authors (Liu and Cohen, 2014;  
378 Garner et al., 2017). Although the tested concentrations (20 and 100  $\mu\text{g/L}$ ) seem then  
379 relatively high from an environmental point of view, they have allowed the recovery of the  
380 contaminant in the exposure media. While most studies focused on behavioral or  
381 physiological traits, molecular parameter assessment is still scarce in aquatic ecotoxicology  
382 despite its sensitivity (Châtel et al., 2018). This is due to the limited genomic information for  
383 sentinel and non-model organisms (Calzolari et al., 2007; Mussali-Galante et al., 2013), such  
384 as bivalve species. Among bivalves, most of the studied organisms are marine species and  
385 mostly represented by *Mytilus galloprovincialis* (Rocha et al., 2015; Revel et al., 2017). The  
386 measurement of expression of known genes for *C. fluminea* is relevant since these genes  
387 might be engaged in key responses against contamination and will give additional information  
388 regarding NP toxicity in freshwater species. In addition, the sensitivity and the precocity of  
389 answer at such a biological level will allow the detection of early warning signals during a  
390 short-term period, as the one applied in this work. This monitoring may also help to provide  
391 knowledge about mechanistic events that can be integrated afterward in AOP approaches for  
392 risk characterization (Revel et al., 2017).

393 As many parameters influenced NP fate in the environment, the measurement of the effective  
394 exposure concentration appeared as a first crucial indication. Our results clearly showed a

395 drop of the concentration of Cu in the water after 24 hours. As usually observed, the loss  
396 might be explained by the process of NP homoaggregation and/or heteroaggregation, which is  
397 enhanced when the concentration increases (Bundschuh et al., 2018; Baker et al., 2014;  
398 Melegeri et al., 2013; Baek and An, 2011; Lead et al., 2018). The fast aggregation kinetic of  
399 NP between the injection and the sampling would be responsible for the lower values  
400 measured than the intended ones (much lower for 100 µg/L treatments). The resulting  
401 sedimentation is then responsible for the reduction of NP concentration in the water. The  
402 adsorption of NP on different abiotic and biotic surfaces can also contribute to this reduction.  
403 Even if the concentrations measured after each renewal were slightly lower than intended  
404 values, they remained close to them. Thus, the daily media renewal ensures that the pressure  
405 of NP exposure was maintained during the whole experiment in a range of concentrations  
406 from 26 to 98% of the intended values depending on the treatment.

407 Bivalves are filter-feeders and are known to bioaccumulate various contaminants  
408 including nanoparticles (Hanna et al., 2014; Rocha et al., 2015, Buffet et al., 2011), but they  
409 were also reported to pack NP in pseudofeces (Montes et al., 2012; Conway et al., 2014) and  
410 in feces (Hull et al., 2011; Ward and Kach, 2009), then reducing the bioaccumulated content.  
411 In our study, neither the digestive gland nor the rest of the soft tissues showed any significant  
412 bioaccumulation. Bioaccumulation of CuO NP was reported in marine bivalves to  
413 concentrations as low as 10 µg/L in *Scrobicularia plana* (Buffet et al., 2011; 2013) and even  
414 after 3 days of exposure in *Mytilus galloprovincialis* (Gomes et al. 2011; 2012; 2013, 2014).  
415 The absence of bioaccumulation recorded in *C. fluminea* remains difficult to explain, but the  
416 short duration of the exposure period might be a first point. Secondly, a short depuration  
417 period was applied. Other authors do not systematically do so and may have overestimated the  
418 actual accumulated concentrations including for example the Cu passing through the lumen of  
419 the digestive tracts. Finally, copper is an essential element that can also lead to toxic effects

420 (Gomes et al., 2011). Bivalves are able to act for its elimination as well as to adopt a  
421 protective behavior such as valve closure or reducing filtration. *C. fluminea* was already  
422 reported to quickly respond to Cu contamination by closing their valves, even at low doses <  
423 5 µg/L (Tran et al., 2003; Castro et al., 2018), which reduced the organism exposure to the  
424 contaminated media. The filtration test we performed clearly showed that clams pre-exposed  
425 to both NP types reduced their filtration activity when further placed in clean water. Besides a  
426 protective response, the CuO NP could have altered the gills (Moëzzi et al., 2018), what may  
427 lead to a reduction of the filtration efficiency. Our experimental design does not enable us to  
428 monitor filtration activity directly during the NP exposure, but we assume that if filtration was  
429 reduced during the exposure (as it was during the test), it might partly explain why significant  
430 accumulation has not occurred. The absence of significant bioaccumulation observed does not  
431 necessarily mean an absence of contact or uptake, as already observed in other studies, using  
432 relatively low exposure concentrations, in which the absence of bioaccumulation of CeO<sub>2</sub> NP  
433 (Koehlé-Divo et al., 2018; Garaud et al., 2015) was measured in bivalve species while strong  
434 biochemical and/or genotoxic effects were highlighted. In order to be sure that there was  
435 effectively no bioaccumulation, the use of labelled NP as tracer should be particularly  
436 relevant since labelling make them easily detectable, even in small quantities (Dybowska et  
437 al., 2011).

438 At the molecular level, the measurement of gene expression allowed us to evidence  
439 significant effects linked to the applied exposure. After 24 and 96 hours, significant effects  
440 were associated to the interaction of both the type and the concentration of the tested NP.  
441 After 24 hours of exposure, the results suggest that 30nm-NP may be less oxidant than 12nm-  
442 NP, because the level of transcripts of genes involved in antioxidant defense was reduced. At  
443 96 hours, an increase of transcripts of detoxification genes was noticed (MT and GSTpi), but  
444 genes of antioxidant defense were not significantly affected. As mentioned in Klaper et al.

445 (2014), the oxidative stress response can dissipate quickly and other molecular pathways such  
446 as drug resistance or detoxification genes can be induced at low exposure concentrations.  
447 Indeed, in the case of exposure to 12nm-NP, it seems that the mechanisms of cellular  
448 excretion (MRP1 and PGP) were elicited, especially after 24 hours at 20  $\mu\text{g/L}$ . Our work  
449 demonstrated for the first time in the clam *C. fluminea* significant but not striking effects of  
450 CuO NP exposure at the molecular level. Coherent pathways were involved in the response  
451 we detected, that was based mainly upon detoxification (30nm-NP) and cellular elimination  
452 (12nm-NP). Our final results showed that the markers investigated at the biochemical level  
453 did not respond to NP contamination regardless of NP type or concentration for both times  
454 monitored. The absence of NP effect at this biological level was already reported in *M.*  
455 *galloprovincialis* exposed to 10  $\mu\text{g}$  Cu/L of CuO NP for 24 hours (Ruiz et al., 2015) and for 3  
456 days (Gomes et al., 2012) but it must be duly noted that detoxification mechanisms (MT,  
457 SOD, CAT, GPx and/or GST) and/or marker of damages (i.e. LPO) were enhanced at a longer  
458 timescale ranging from 7 to 21 days (Gomes et al., 2012; Ruiz et al., 2015). A same trend was  
459 observed in *C. fluminea* exposed to 10 and 100  $\mu\text{g/L}$  of CeO<sub>2</sub> NP where none of the  
460 monitored biochemical markers have been modified after 2 days of exposure but significant  
461 damages (i.e. apoptosis) were observed after 6 days of exposure (Koehl -Divo et al., 2018).

462 The different responses monitored in this study for both tested NP may be explained by their  
463 differing sizes and specific surface areas. Because of their smaller size, 12nm-NPs display a  
464 higher specific surface area. Consequently, for a given mass, the surface of 12nm-NP  
465 available to interact with the organisms will be larger. Per mass unit, the exposure  
466 concentrations are similar, but once normalized by the specific surface area, the exposure  
467 concentrations are twice as high for 12nm-NP. In addition, the difference of particle size may  
468 lead to a higher amount of 12nm-NP particles per mass concentration than 30nm-NP (Quik et  
469 al., 2014; Singh et al., 2009). Moreover, the kinetics of transformation as dissolution are

470 expected to be faster for 12nm-NP because of their higher specific surface area (Auffan et al.,  
471 2009; Singh et al., 2009). Although their uncoated surfaces may have led to their fast  
472 aggregation in the exposure media, and by this, to the reduction of their surface reactivity,  
473 their interactions with the biological surfaces should allow their disaggregation through  
474 mechanical actions (gill cilia, labial palps) and/or their dissolution due to the composition  
475 change (proteins, pH) (Rocha et al., 2015; Ward and Kach, 2009; Canesi et al., 2012, Griffitt  
476 et al., 2008). The high sensitivity of *C. fluminea* to copper could perhaps explain their  
477 physiological responses and the entry of CuO NPs and ions in the cells could have also  
478 contributed to the observed responses by interacting with cellular components. Finally, our  
479 results are in agreement with those obtained in the literature which have shown different  
480 effects of NP according to their size on ecotoxicological endpoints, the smaller being usually  
481 more toxic than the bigger ones (Hu et al., 2014; Peng et al., 2017).

## 482 **Conclusion**

483 In this study, the short-term exposure of the Asian clam *C. fluminea* to relatively low  
484 concentrations of NPs (20 & 100 µg CuO/L) compared to the available literature highlighted  
485 significant modifications at different scales of biological organization from physiological to  
486 molecular levels. Integrated gene expression analysis revealed a different mechanism of  
487 action depending on NP treatment, with one enhancing antitoxic related mRNAs while the  
488 other presented gene expression modification linked to cellular elimination pathway. This  
489 analysis was performed on a restricted number of genes due to the lack of gene sequences in  
490 the clam *C. fluminea*. The filtration rate appeared also useful for the assessment of NP toxicity  
491 and induced an avoidance response of *C. fluminea* by valve closure. On a longer time scale,  
492 the reduction of the filtration rate and the gene expression modification could be increased  
493 and also lead to further side-effects. In the future, with the next generation sequencing  
494 advances, the omic tool will allow a screening of a larger set of genes involved in several  
495 pathways, like cell proliferation, cell signaling, or DNA repair. Although *C. fluminea* is  
496 considered as relatively tolerant to contaminants compared to other bivalves (Castro et al.,  
497 2018), these NPs were shown here as reactive in a short-term exposure and using a  
498 waterborne contamination. In regard to our results, the assessment of these NP impacts on *C.*  
499 *fluminea* should be addressed in more complex as well as in longer timescale experiments  
500 which can be achieved by the setting up of mesocosm experiments. The multi-scale approach  
501 is particularly relevant for a better evaluation and understanding of a potential toxicity on  
502 individuals and contribute to increased information regarding the first steps of an AOP  
503 approach. Such an assessment should be further developed at higher biological levels,  
504 including population and communities.



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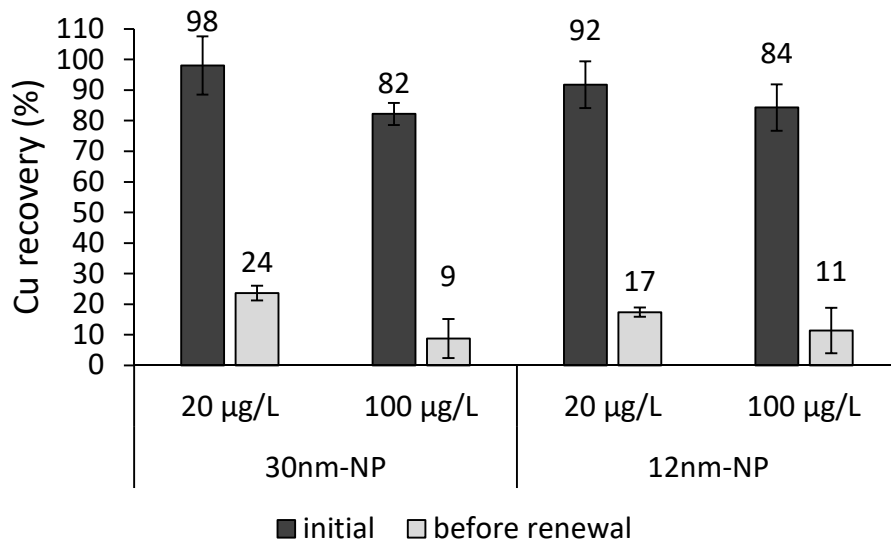


Figure 1: Percentages of Cu recovery compared to nominal Cu concentrations injected in the water column for each CuO NP treatment. These measurements are the mean  $\pm$  SD (n=3) of the initial concentrations (initial) and concentrations after 24 hours (before renewal) for each NP treatment, obtained from samples collected at the start and after 24 and 72 hours of experiment (See §2.4).

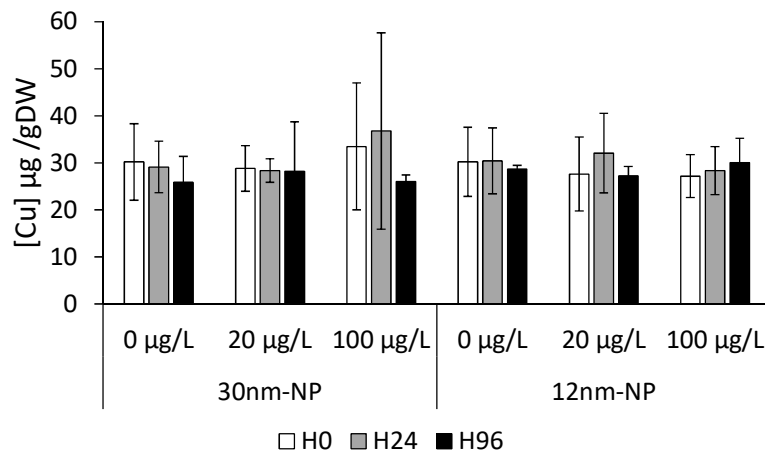


Figure 2: Total Cu concentrations (mean  $\pm$ SD, n=5) in the digestive gland of *C. fluminea* exposed to two different CuO NPs during 0, 24 and 96 hours. No significant differences between groups were pointed out by statistical tests.

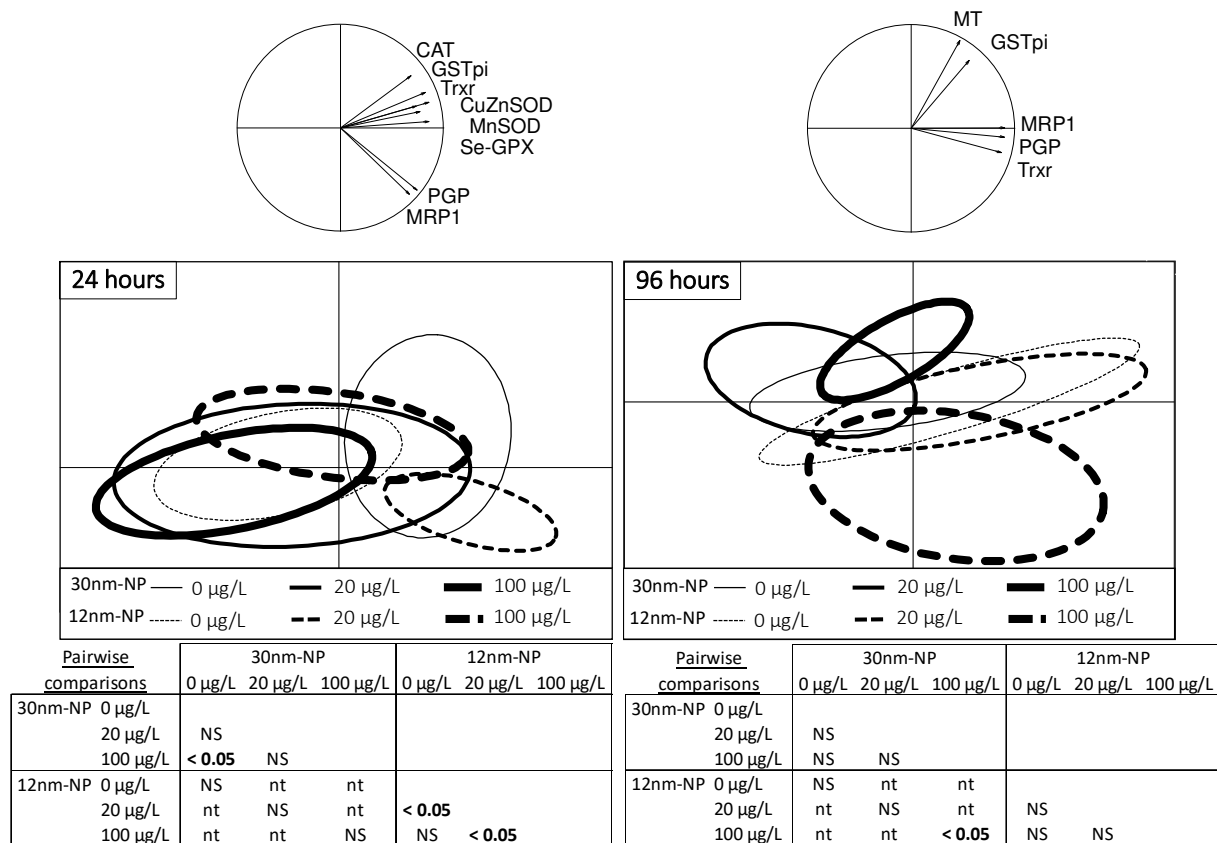


Figure 3: PLS-DA analyses using gene expressions (See table S1) monitored in *C. fluminea* digestive gland after 24 hours (left side) and 96 hours (right side) of exposure to different CuO NP (30nm-NP & 12nm-NP) at different concentrations (0, 20 and 100 µg CuO/L). The factorial plane represents expression differences between exposure groups while the correlation circle shows important variables in the projection having VIP > 0.8. The table associated with each PLS-DA shows the pairwise comparisons results. Bold values correspond to significant differences, “NS” are non-significant values and “nt” are non-tested comparisons. Quantitative PCR data were normalized using the β-actin gene.

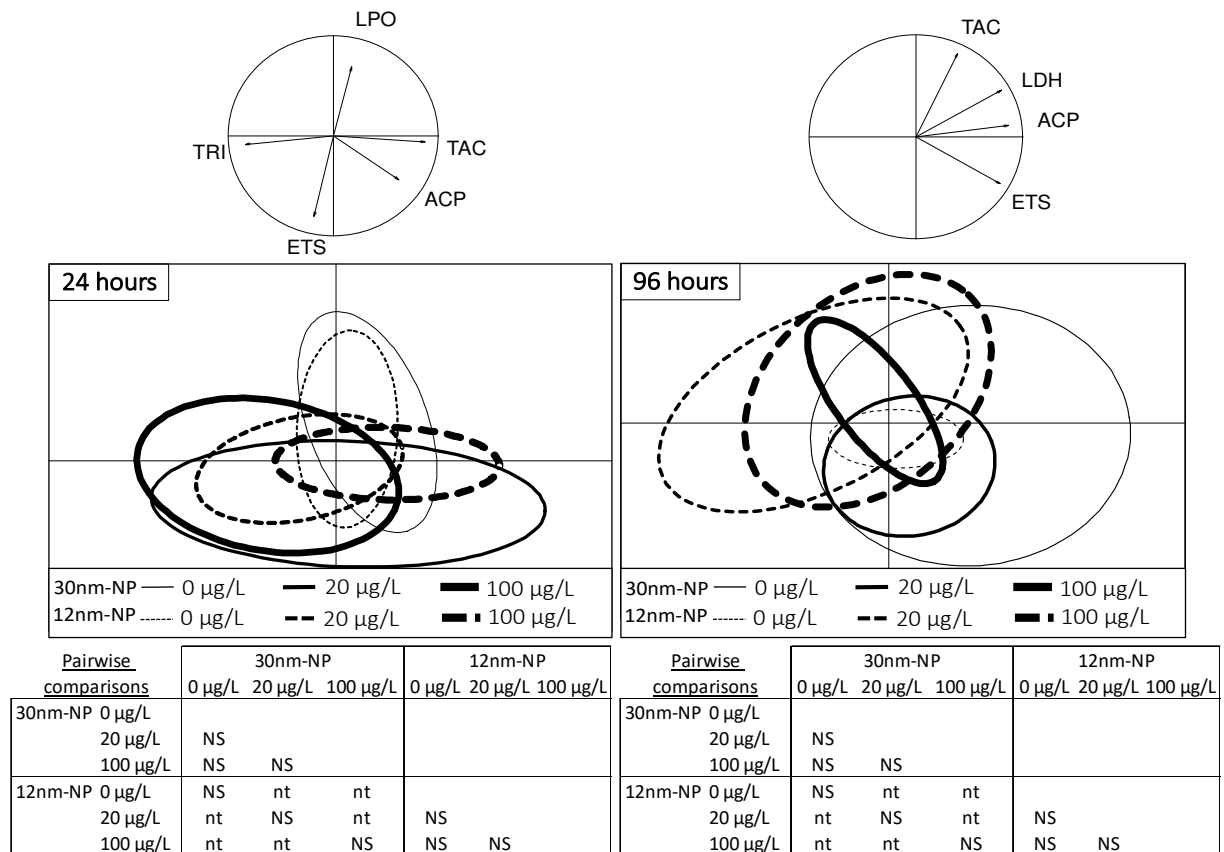


Figure 4: PLS-DA analyses using biochemical biomarkers (See §2.6) measured in *C. fluminea* digestive gland after 24 hours (left side) and 96 hours (right side) of exposure to different CuO NP (30nm-NP & 12nm-NP) at different concentrations (0, 20 and 100 µg CuO/L). The factorial plane represents biomarkers response related to exposition groups while the correlation circle shows important variables in the projection having VIP > 0.8. The table associated with each PLS-DA shows the pairwise comparisons results. Bold values correspond to significant differences, “NS” are non-significant values and “nt” are non-tested comparisons.

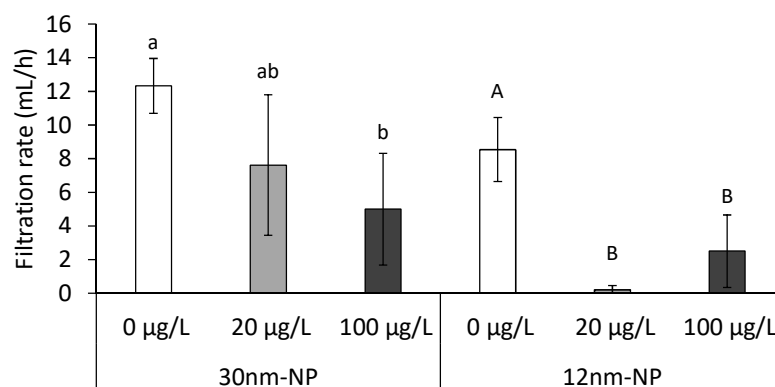


Figure 5: Filtration rate of *C. fluminea* exposed for 96 hours to two different CuO NPs (30nm-NP & 12nm-NP) at three concentrations (0, 20 and 100 µg/L). The presented results are mean ± SD). Groups with different letters (lowercase for 30nm-NP, capital for 12nm-NP) are significantly different.

Control  
12nm CuO NP  
30nm CuO NP



**24 and 96 hours**  
0, 20 and 100 µg CuO/L

- ⇒ **No Cu bioaccumulation**
  - ⇒ **Biochemical markers**  
*(no response)*
  - ⇒ **Gene expressions**  
*(modulation)*
  - ⇒ **Filtration rate**  
*(reduction)*
- Different response according to NP size*