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

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Abstract

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

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

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

1. Introduction

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

In the framework of nanotoxicology, the assessment of NP toxicity according to their physico-chemical properties remains a work in progress. Moreover, their biocide abilities raise the question of their (eco)toxicity towards non-target organisms (Keller et al., 2017). In aquatic environments, Cu-based NPs would be accumulated in the sediments of freshwater and marine environments (Keller et al., 2017). Benthic species should be of particular concern because of their close contact with contaminants and because their burrowing activities leads to a potential remobilization of contaminants (Roberts, 2012). The widespread endobenthic freshwater bivalve *Corbicula fluminea* is a good model for such assessments due to its ability to be both filter- and pelagic-feeder, but also due to its ability to strongly bioaccumulate a large amount of contaminants, such as metals (Inza et al., 1997; Shoults-Wilson et al., 2010; 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 galloprovinciallis* 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^{2+}.

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

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

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

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

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

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

2. Materials and methods

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

2.1. Tested Nanoparticles:

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

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

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

2.2. Collection and acclimatization of organisms:

Freshwater Asian clams *C. fluminea* (3.1 ± 0.3 cm) were hand-collected in June 2016 in the Moselle River, La Maxe (49°09'42.9"N 6°11'55.0"E, France) at a water temperature of 21°C. The physico-chemical characteristics of the site water were previously measured (NH_4: 0.03 mg N/L; NO_2: < 0.05 mg N/L; NO_3: 1.69 mg N/L; PO_4: 0.01 mg P/L; P tot: 0.10 mg P/L; Cl^-: 214 mg/L; SO_4: 52 mg/L; TAC: 2.3 meq; MEST: 15.4 mg/L; MVS: 37.2%; Ca^{2+}: 117 mg/L; $Mg^{2+;}$ 9.3 mg/L; Na^+: 61.4 mg/L; K^+: 4.2 mg/L; DCO: 52 mg/L; DBO5: 4 mg/L; Tot A: 9.54 meq/L; Tot C: 9.4 meq/L; Bal.: -0.7%; pH: 8.0; Cond: 1040 µS/cm; dissolve Cu: 1 µg/L; Cu tot.: 1.7 µg/L). After sampling, the clams were transported to the laboratory in their water of origin in plastic coolers. They were acclimated progressively to the experimental conditions in a temperate room (15 °C) for 3 days by gradually increasing the proportion of artificial water that will be used for the experiment. The artificial water was prepared using commercial salt (Tropic Marin®, Tropicarium Buchshlag Dreieich, Germany) diluted in DIW at 1.5 psu. The elemental composition of this salt (major cations, major anions, nutrients and trace elements) was assessed by Atkinson and Bingman (1997). The media was continuously aerated and a natural photoperiod was kept (16h light: 8h dark). One day before the experiment, two organisms per beaker were placed in 200 mL of water in order to acclimate them to the device.

2.3.Experimental design

C. fluminea were exposed to 0 (controls), 20 and 100 µg CuO/L of each NP (30nm-NP and 12nm-NP) for 96 hours at 15°C in artificial water (1 *C. fluminea*/100 mL). The consideration of the SSA of each NP lead to 0.000506 m²/L and 0.00253 m²/L for 30nm-NP at 20 µg/L and 100 µg CuO/L, respectively, and 0.00094 m²/L and 0.0047 m²/L for 12nm-NP at 20 µg/L and 100 µg CuO/L, respectively. The water was renewed every day. No aeration and no feeding

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

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

2.4.Measurements of water and tissue copper concentrations

As the media was changed daily, the water was sampled for total Cu measurement immediately after NP introduction and after 24 hours (just before the next renewal) in 3 beakers per treatment and time selected randomly. This procedure was applied three times: at the start of the exposure and at the renewal at 24 and 72 hours of exposure. Five clams per treatment were dissected after 24 hours of depuration at each time step (0, 24, 96 hours) for Cu measurement. Then, the digestive gland and the remaining soft tissues were selected, freeze-dried, weighed and digested in 69% HNO_3 for 24 hours at 60°C. Concentrations were determined by flame for concentrations above 10µg Cu/L (Perkin-ELMER Aanalyst 100) or by graphite furnace for concentrations below 10 µg/L (VARIAN Spectraa 800 with ZEEMAN correction) atomic absorption spectrophotometry for each individual clam. Certified water (SRM 3114) was used to check the accuracy of the quantification for the water samples (20.2 ± 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 ± 2.6 µg Cu/g for 106 ± 10
223 µgCu/g). Metal concentration in clams are expressed as $\mu g.g^{-1}$ dry weight (DW) tissue and
224 $\mu g.L^{-1}$ 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 later 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 Bioanalyseur 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 synthetized 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

verified on agarose gel. All PCR amplifications were performed on each biological replicate using the StepOnePlus RT-PCR system (Applied Biosystems®). Gene expression levels were analyzed using the relative quantification method (ΔΔCt) (Livak and Schmittgen, 2001). Two genes were selected as potential normalizing genes (S3 and β-actin), but only β-actin has revealed sufficient stability in our exposure conditions. β-actin was then used as a reference for normalization. The control condition (0 µg/L) was chosen as the reference condition. The final treated/control ratio and the pooled standard deviation were calculated based on the mean ΔCt of the biological replicates. Statistical analyses were performed separately for each exposure duration (24 & 96 hours) as described below (§2.8).

2.6. Biochemical biomarker analysis

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

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

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

2.7. Filtration rate measurements

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

2.8. Statistical analysis

Statistical analyses were conducted using R (R Core Team, 2014). For the filtration rate and the bioaccumulation results, the homoscedasticity and the normality were checked by Levene 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 ± 0.38 µg 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 µg CuO/L treatments with 98 ± 10 % and 92 ± 8 % recovery for
316 30nm-NP and 12nm-NP, respectively. High recoveries were also measured for both 100 µg
317 CuO/L treatments with 30nm-NP and 12nm-NP presenting 82 ± 4 % and 84 ± 8 % recovery,
318 respectively. Cu concentrations were also measured before each renewal (after 24 hours) and
319 showed a decrease of 74 ± 1 % in all NP treatments (20 & 100 µg/L) after 24 hours in the test
320 media.

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

3.2 Gene expression

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

The Manova analysis conducted on the 96 hour gene expression dataset indicated a significant impact of NP type alone ($F= 17_{2\text{-}29\ df}$, $p < 0.001$) and of its interaction with NP concentration ($F= 4.1_{4\text{-}60\ df}$, $p < 0.01$) while no significant difference was shown for the concentration effect alone ($F= 1.1_{4\text{-}60\ df}$, $p = 0.37$). The pairwise comparisons (fig. 3, table right side) only showed a clear discrimination between both NP types (30nm-NP & 12nm-NP) at the highest exposure concentration (100 µg/L). The associated PLS-DA is presented in fig. 3 (right side). Both NP types at 100 µg/L were separated by the vertical axis that showed a higher mRNA content of 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: $F = 0.45_{2\text{-}29\ df}$, $p = 0.65$; 96h: $F = 0.2.9_{2\text{-}29\ df}$, $p = 0.07$), NP type (24h: $F= 1.95_{4\text{-}60\ df}$, $p= 0.11$; 96h: $F= 1.99_{4\text{-}60\ df}$, $p= 0.11$) or
357 interaction between them (24h: $F = 0.1.67_{4\text{-}60\ df}$, $p = 0.17$; 96h: $F = 0.86_{4\text{-}60\ df}$, $p = 0.49$). 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 µg CuO/L) and the ones exposed to 12nm-NP
365 presented a significant decrease of filtration activity for both exposure concentrations (20 &
366 100 µg 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 µg/L and can even reach 20 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 µg/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 µg/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 (Calzolai 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

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

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

(Gomes et al., 2011). Bivalves are able to act for its elimination as well as to adopt a protective behavior such as valve closure or reducing filtration. *C. fluminea* was already reported to quickly respond to Cu contamination by closing their valves, even at low doses < 5 µg/L (Tran et al., 2003; Castro et al., 2018), which reduced the organism exposure to the contaminated media. The filtration test we performed clearly showed that clams pre-exposed to both NP types reduced their filtration activity when further placed in clean water. Besides a protective response, the CuO NP could have altered the gills (Moëzzi et al., 2018), what may lead to a reduction of the filtration efficiency. Our experimental design does not enable us to monitor filtration activity directly during the NP exposure, but we assume that if filtration was reduced during the exposure (as it was during the test), it might partly explain why significant accumulation has not occurred. The absence of significant bioaccumulation observed does not necessarily mean an absence of contact or uptake, as already observed in other studies, using relatively low exposure concentrations, in which the absence of bioaccumulation of CeO_2 NP (Koehlé-Divo et al., 2018; Garaud et al., 2015) was measured in bivalve species while strong biochemical and/or genotoxic effects were highlighted. In order to be sure that there was effectively no bioaccumulation, the use of labelled NP as tracer should be particularly relevant since labelling make them easily detectable, even in small quantities (Dybowska et al., 2011).

At the molecular level, the measurement of gene expression allowed us to evidence significant effects linked to the applied exposure. After 24 and 96 hours, significant effects were associated to the interaction of both the type and the concentration of the tested NP. After 24 hours of exposure, the results suggest that 30nm-NP may be less oxidant than 12nm-NP, because the level of transcripts of genes involved in antioxidant defense was reduced. At 96 hours, an increase of transcripts of detoxification genes was noticed (MT and GSTpi), but 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 µg/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 µg 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 µg/L of CeO_2 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 (Koehlé-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

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

Conclusion

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

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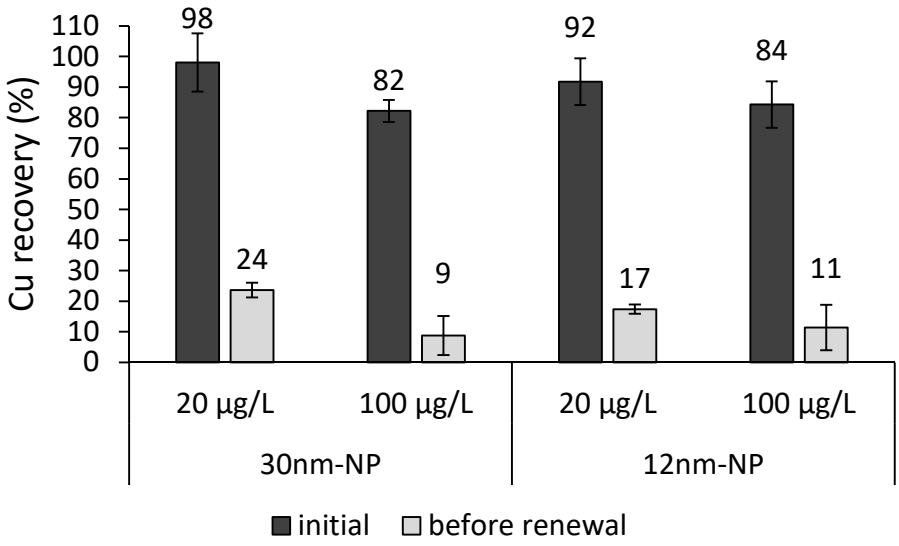


Figure 1: Percentages of Cu recovery compared to nominal Cu concentrations injected in the water column for each CuO NP treatment. These measurements are the mean ± SD (n=3) of the initial concentrations (initial) and concentrations after 24 hours (before renewal) for each NP treatment, obtained from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

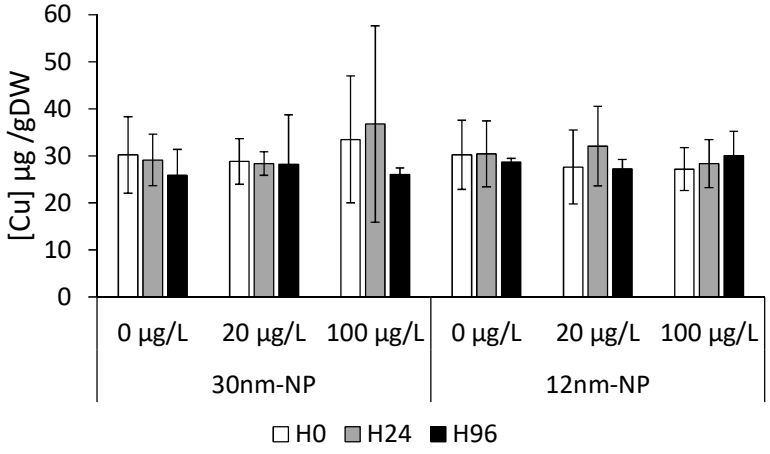
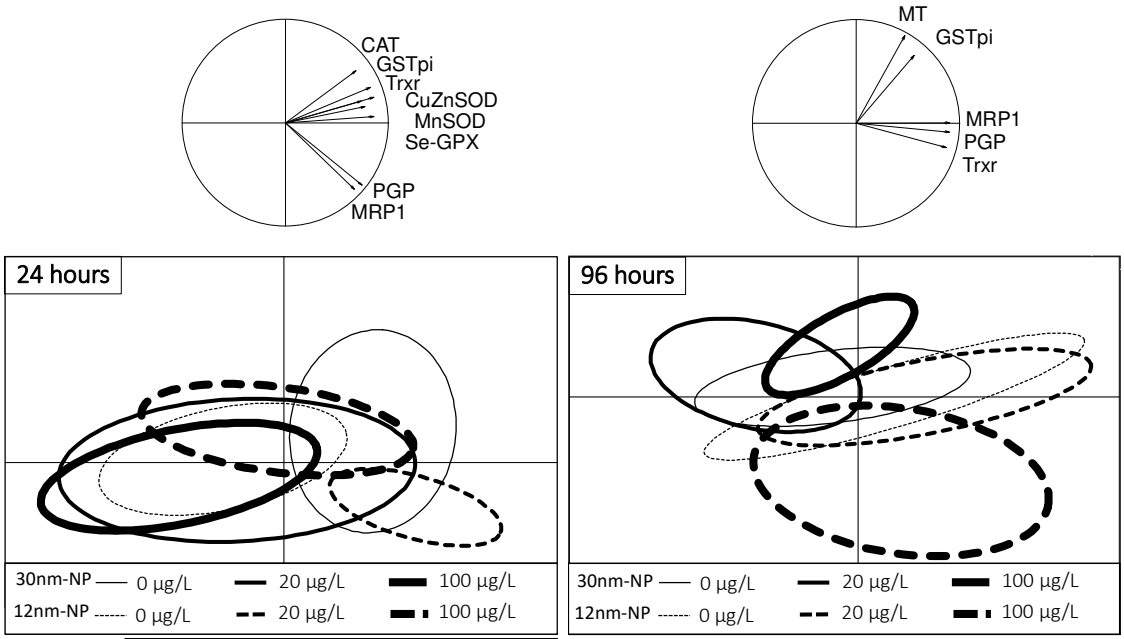


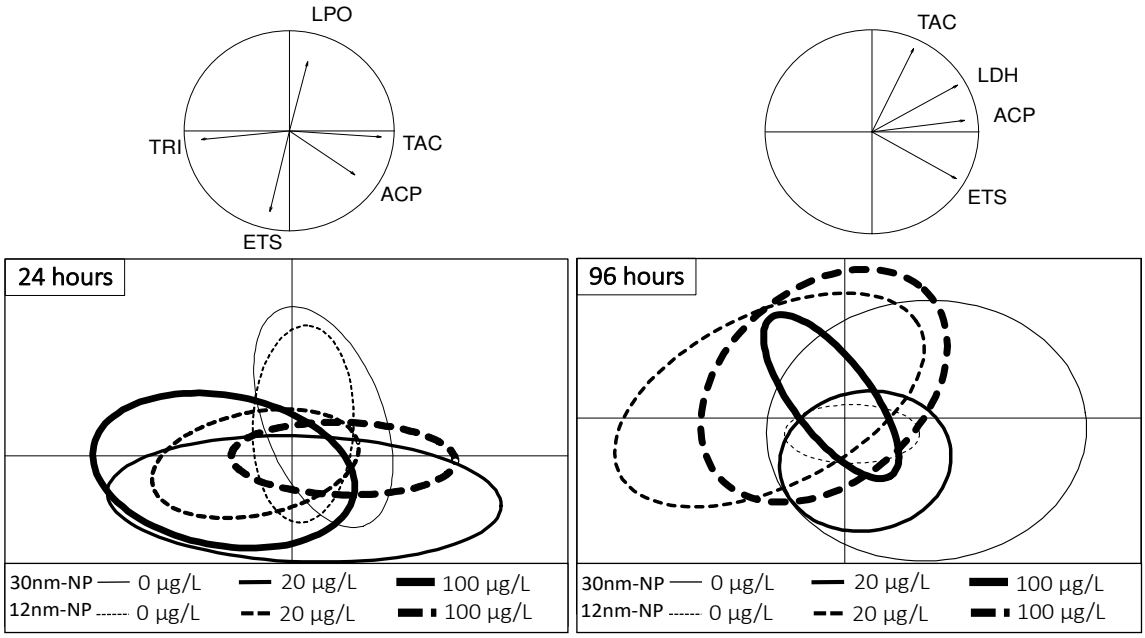
Figure 2: Total Cu concentrations (mean ±SD, n=5) in the digestive gland of *C. fluminea* exposed to two different CuO NPs during 0, 24 and 96 hours. No significant differences between groups were pointed out by statistical tests.



Pairwise comparisons		30nm-NP 0 µg/L	30nm-NP 20 µg/L	30nm-NP 100 µg/L	12nm-NP 0 µg/L	12nm-NP 20 µg/L	12nm-NP 100 µg/L
30nm-NP	0 µg/L						
	20 µg/L	NS					
	100 µg/L	**< 0.05**	NS				
12nm-NP	0 µg/L	NS	nt	nt			
	20 µg/L	nt	NS	nt	**< 0.05**		
	100 µg/L	nt	nt	NS	NS	**< 0.05**	

Pairwise comparisons		30nm-NP 0 µg/L	30nm-NP 20 µg/L	30nm-NP 100 µg/L	12nm-NP 0 µg/L	12nm-NP 20 µg/L	12nm-NP 100 µg/L
30nm-NP	0 µg/L						
	20 µg/L	NS					
	100 µg/L	NS	NS				
12nm-NP	0 µg/L	NS	nt	nt			
	20 µg/L	nt	NS	nt	NS		
	100 µg/L	nt	nt	**< 0.05**	NS	NS	

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.



Pairwise comparisons		30nm-NP 0 µg/L	30nm-NP 20 µg/L	30nm-NP 100 µg/L	12nm-NP 0 µg/L	12nm-NP 20 µg/L	12nm-NP 100 µg/L
30nm-NP	0 µg/L						
	20 µg/L	NS					
	100 µg/L	NS	NS				
12nm-NP	0 µg/L	NS	nt	nt			
	20 µg/L	nt	NS	nt	NS		
	100 µg/L	nt	nt	NS	NS	NS	

Pairwise comparisons		30nm-NP 0 µg/L	30nm-NP 20 µg/L	30nm-NP 100 µg/L	12nm-NP 0 µg/L	12nm-NP 20 µg/L	12nm-NP 100 µg/L
30nm-NP	0 µg/L						
	20 µg/L	NS					
	100 µg/L	NS	NS				
12nm-NP	0 µg/L	NS	nt	nt			
	20 µg/L	nt	NS	nt	NS		
	100 µg/L	nt	nt	NS	NS	NS	

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.

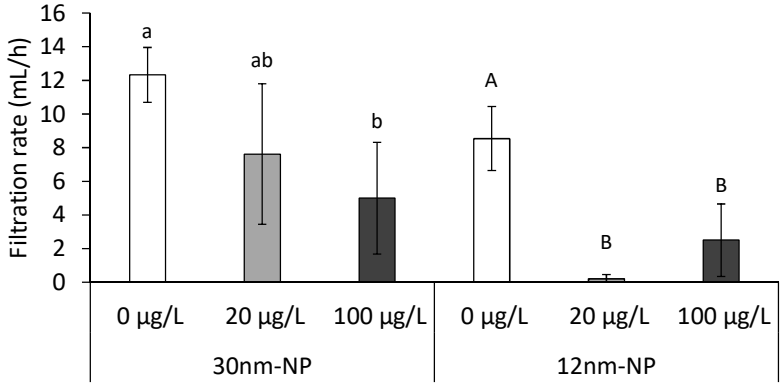
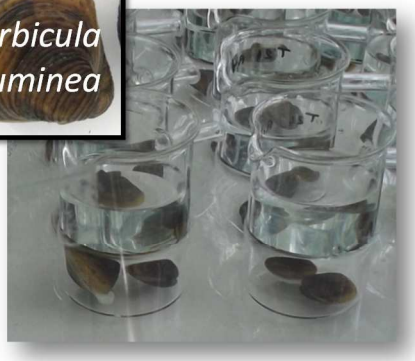


Figure 5: Filtration rate of *C. fluminea* exposed for 96 hours to two different CuO NPs (30nm-NP & 12nm-NP) at three concentrations (0, 20 and 100 µg/L). The presented results are mean ± SD). Groups with different letters (lowercase for 30nm-NP, capital for 12nm-NP) are significantly different.

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