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Behavioural and biochemical responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles

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ABSTRACT

Engineered nano-sized Cu oxide particles are extensively used in diverse applications. Because aquatic environments are the ultimate "sink" for all contaminants, it is expected that nanoparticles (NP) will follow the same fate. In this study, two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* were chosen as ecotoxicological models. The aim was to evaluate behavioural (burrowing kinetics, feeding rate) and biochemical (biomarkers) responses of *S. plana* and *H. diversicolor* exposed in the laboratory to Cu (10 μg L^{-1}) added in natural seawater either in the form of engineered nanoparticles (NPs) of CuO or as dissolved Cu in 2% HNO_3. Exposure was characterized by considering (i) the physico-chemical fate of NP (ii) the fraction of labile Cu in experimental media and (iii) Cu bioaccumulation. Results showed high aggregation of CuO NPs in seawater and no additional bioavailable Cu concentrations. Behavioural impairments were observed in *S. plana* exposed to CuO NPs or soluble Cu whereas in *H. diversicolor*, only the exposure to soluble Cu led to a burrowing decrease. No obvious neurotoxicity effects were revealed since in both species, no changes in cholinesterasic activity occurred in response to both forms of Cu exposure. Biomarkers of oxidative-stress catalase and glutathione-S-transferase were enhanced in both species whereas superoxide dismutase was increased only in *S. plana* exposed to CuO NPs. Metallothionein-like protein was increased in bivalves exposed to both forms of Cu. Since, no detectable release of soluble Cu from CuO NPs occurred during the time of experiment, ecotoxicity effects seem to be related to CuO NPs themselves.

1. Introduction

Because the physicochemical properties of nanoparticles are distinct from their bulk counterparts, the fast growth of nanotechnologies has brought new industrial and business opportunities. For instance, engineered nano-sized copper oxide particles (CuO NP) are commonly used as bacteriocides and have the potential to replace noble metal catalysts for carbon monoxide oxidation (Zhou et al., 2006). CuO NP suspensions (nanofluids) have excellent thermal conductivity and are used as a heat transfer fluid in machine tools (Chang et al., 2005). Health and environmental concerns on free engineered nanoparticles (ENPs) have been highlighted in several reports (Royal Commission on Environmental Pollution, 2008; European Commission, 2009). Because aquatic environments are the ultimate "sink" for all contaminants, it is expected that ENPs will follow the same fate (Kaegi et al., 2008). Environmental monitoring data of engineered NPs, arising from use in consumer products, are currently lacking, which could be due to the difficulty to detect and quantify ENPs in complex matrices, such as water, sediments, and soils, as well as in organisms and their susceptible tissues (Wiesner et al., 2006; Nowack and Bucheli, 2007). Most of the ecotoxicological studies published are conducted with high NP concentrations, probably unrealistic from

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an environmental point of view as indicated by Predicted Environmental Concentrations of ENPs arising from use in consumer products (Tiede et al., 2009).

In order to investigate putative ecological impairments caused by ENPs, it is necessary to select species which have a key role in the structure and functioning of ecosystems and are recognized as good models for biomonitoring purposes. In this way, marine invertebrates such as the ragworm *Hediste diversicolor* and the bivalve mollusc *Scrobicularia plana* (Byrne and Halloran, 2001; Solé et al., 2009) represent good candidates. Soluble forms of copper are highly toxic to aquatic organisms (Eisler, 2007; Luoma and Rainbow, 2008). In the case of Cu-containing NPs, the release of Cu and its speciation may be a key factor in their ecotoxicity, besides that related to the nanoparticle itself. Biomarkers of defence metallothionein: MT, catalase: CAT, glutathione-S-transferase: GST, superoxide dismutase: SOD and damage thibarbyturic acid reactive substances: TBARS, cholinesterase: ChE are commonly used in environmental assessment (Lagadic et al., 2000; Amiard and Amiard-Triquet, 2008). Lactate dehydrogenase (LDH) activity, as the terminal enzyme of anaerobic glycolysis, plays an important function in anaerobic metabolism (Gagnon and Holdway, 1999; Diamantino et al., 2001). Clams (*S. plana*) are able to maintain a working anaerobic metabolism under anoxia and hydrogen sulphide exposure (Oeschger and Pedersen, 1994). Induction in LDH activity due to chemical stress has been reported in different species of bivalves such as *S. plana* (Boldina-Cosqueric et al., 2010), *Perna viridis* (Nicholson and Lam, 2005), *Donax trunculus* (Tlili et al., 2010) and polychetes such as *H. diversicolor* (Moreira et al., 2006). In addition, behavioural biomarkers are sensitive tools to assess the impact of the contaminants at concentrations far below the lethal effect (Amiard-Triquet, 2009).

Nanoparticles of CuO were identified has being important in ecotoxicological assays due to their relatively low dissolution rate but their potentially high toxicity towards organisms (Stone et al., 2010). Thus, the aim of the present study was to evaluate biochemical and behavioural responses of two marine endobenthic invertebrates, *S. plana* and *H. diversicolor*. We selected an aqueous medium because it was difficult to distribute evenly and reliably NPs in a natural sediment medium and both intra-sedimentary species studied were able to freely contact with pollutants in the aqueous medium. Indeed, *H. diversicolor* is a hemisessile polychaete living in galleries aerated by water fluxes generated by body movement. It creates a burrowing environment that may be relatively independent of the surrounding sediment since the burrow is coated by the secretion of a mucus layer. In the case of *S. plana*, the cavity oxygenate water is flushed through the burrow from time to time and within the animal the soft tissues are bathed in pallial fluid with is primarily seawater. Both species were exposed in the laboratory to environmentally relevant Cu levels (10 $\mu g\ L^{-1}$) added in natural filtered seawater either in the form of engineered CuO NP or as soluble Cu. This Cu level (10 $\mu g\ L^{-1}$) was reported in marine environments highly contaminated by this metal (Bryan and Langston, 1992). Exposure was characterized by considering the fate of NP (size distribution and fraction of labile Cu in experimental media) and Cu bioaccumulation.

2. Material and methods

2.1. Chemicals used and particle characterization

CuO nanopowder was obtained from a commercial source (Intrinsiq Materials Limited) with the particle size specified by the manufacturer as 10–100 nm. A stock suspension of CuO nanoparticles (25 $mg\ L^{-1}$) was prepared in deionized water (DIW), stored at 20 °C and used for testing within 2 weeks. Before use, this stock suspension was sonicated for 5 min (Ultrasonicator BRANSON B-1200 E2 HF 100 W). A stock solution of dissolved Cu as $CuNO_3$ (1 g Cu L^{-1}, in 2% HNO_3 w/w) was purchased from Fluka Analytical. All the other chemicals were purchased from Sigma Aldrich.

The stability of CuO NP in both DIW (stock solution used within 2 weeks after preparation) and in the experimental medium (natural seawater) in the absence ($t = 0$) and presence ($t = 2$ d) of organisms was monitored by dynamic light scattering (DLS). A suspension of CuO nanoparticles (25 $mg\ L^{-1}$) in DIW was ultrasonicated for 10 s and the hydrodynamic diameter of the particles was measured using DLS (Malvern, Zetasizer Nano ZS). Then, 5 μL of this stock solution were spiked into natural seawater (10 $\mu g\ L^{-1}$ Cu final concentration), and subsequently the size distribution and zeta potential of CuO nanoparticles were measured.

2.2. Animal collection and acclimatation

Worms (*H. diversicolor*) and bivalves (*S. plana*) were collected by hand from an intertidal mudflat (upper 20 cm depth) in the Bay of Bourgneuf (2°04′40.60″W, 46°56′23.08″N), located on the West Atlantic coast (France), in July 2009. This site is monitored by the French "Mussel Watch" Programme (Réseau National d'Observation, 2006) and is documented as relatively clean (Kalman et al., 2009). Only bivalves with shell length ranging from 15 to 20 mm were selected to avoid any potential influence of sexual maturity. Worms from the same size were selected to avoid a potential influence of weight (0.40 ± 0.13 g mean wet weight) and they were collected in June which did not correspond to the period of sexual maturity (Mouneyrac et al., 2010). Then, *H. diversicolor* and *S. plana* were transported to the laboratory in cool boxes with sediment from the collection site. In the laboratory, animals were allowed to eliminate their gut contents and acclimatize for 48 h in aerated natural seawater, UV treated and filtered through 0.45 μm. This acclimatization time period was chosen after Poirier et al. (2006) and Burlinson and Lawrence (2007).

2.3. Exposure protocol

Bivalves (*S. plana*) were placed into 2.2 L polypropylene aquaria (12 individuals/tank) filled with 2 L seawater and worms (*H. diversicolor*) were introduced individually in plastic beakers of 100 mL filled with 50 mL seawater. Three treatments: (i) natural seawater only; (ii) CuO NPs: 10 $\mu g\ Cu\ L^{-1}$; and (iii) dissolved Cu: 10 $\mu g\ Cu\ L^{-1}$, were carried out in a triplicate design (three tanks per treatment for each species) during 16 d for *S. plana* and 7 d for *H. diversicolor*, using a semi-static exposure regime, in the dark at the temperature they experienced in their sediment of origin at this period of the year (21 °C). The experimental media (water and contaminant) were renewed every other day. In order to avoid interferences between food and the fate of NPs, invertebrates remained unfed during the whole experiment. Previous studies carried out in our laboratory have shown that fast did not induce any decrease of the condition index of clams or histopathological changes for both species.

2.4. Metal quantification in experimental medium

The estimation of dissolved fraction of Cu was determined during *S. plana* exposure in laboratory to seawater only (Controls), soluble Cu or CuO NP, by using DGT tools (diffusive gradients in thin films) (Davison and Zhang, 1994).

This technique, based on mass transport control of the chemical species of interest from water or sediment pores water, uses two hydrogel layers. A polyacrylamide gel is used as the diffusive layer, and is backed up with a second thin gel layer containing a Chelex

cation-exchange resin selective for trace metals. The diffusive layer of known thickness is placed in the DGT probe on top of the binding phase and covered with a filter used to avoid biofouling. Ions diffuse through the filter and diffusive layer to reach the Chelex resin. The mass of the diffused ion, M, can be obtained by direct measurement of the ion concentration (C_r) in the resin layer with total volume of resin V_r:

$$M = C_r V_r.$$

The DGT disc units (2.5 cm diameter corresponding to a 3.14 cm^2 diffusive area) were purchased from DGT Research Ltd. A Chelex-100 resin beads and a diffusive gel with a pore size of about 5 nm were used (open pore diffusive gel) (Zhang and Davison, 1999). The thickness of gel was 0.82 mm. A filter of 0.14 mm thickness and 0.45 μm pores size covered the gel.

For each condition, 8 DGT units were deployed in the tanks containing *S. plana* individuals and placed in temperature controlled room (22 °C). In order to avoid a bias due to the presence of DGT units in the medium, these individuals were not employed for biological tests.

Measurement of metal accumulated by the DGT units was realised on day 0, 3, 7 and 16 (2 DGT per sampling day). After sampling, DGT units were washed with ultra-pure water (Milli-Q) and placed in 1.5 mL vials. One millilitre of 1 M HNO_3 was added and left for a day to elute copper from the Chelex resin. Copper determination was performed by graphite Furnace Atomic Absorption Spectrometry (Varian SpectrAA 800) in either neat or diluted samples of the 1 M HNO_3 eluent. Mass of metal accumulated (M) by the DGT unit was calculated as follows:

$$M = C_r V_r = C_e(V_g + V_e)/f_e$$

where C_e is the Cu concentration in HNO_3 (μg L^{-1}), V_g and V_e, the volumes of the gel ($0.15.10^{-3}$ L) and the eluent (1.10^{-3} L) respectively and f_e, the elution factor (0.8).

2.5. Biochemical markers

For each biomarker, 10 bivalves were collected from the three different tanks ($n = 3$ or $n = 4$ taken randomly from each tank) corresponding to each experimental condition. After exposure, the length and the total weight of bivalves were recorded. After removal of the shells, the soft tissues were carefully wiped with absorbent paper and stored at −80 °C until biochemical analysis. For worms, following exposure, they were carefully wiped with absorbent paper, weighed individually and stored at −80 °C until biochemical analysis. For MTLP and LDH, measurements were carried out individually ($n = 10$ for each biomarker). For GST, CAT, SOD, TBARS and ChE, determinations were carried out on 5 pools of 2 individuals per species (*H. diversicolor*, *S. plana*) per treatment.

Because previous studies revealed that metallothionein-like protein (MTLP) determination was not a relevant biomarker of metal exposure in *H. diversicolor* (Poirier et al., 2006), MTLP analyses were performed only in bivalves (*S. plana*). The whole soft tissues of *S. plana* were homogenised at 4 °C in 20 mM TRIS, 10^{-5} mM β-mercaptoethanol, 0.1 mM Phenylmethanesulfonyl Fluoride (PMSF), 150 mM NaCl adjusted to pH = 8.6 (4 mL g^{-1} soft tissue). The soluble (S1) and insoluble (P1) fractions were separated by centrifugation at 30 000*g* for 30 min at 4 °C. An aliquot of the soluble fraction (S1) was heated at 75 °C for 15 min. Then MTLPs (i.e. heat-stable thiolic compounds) were isolated by centrifugation (15 000*g* for 10 min at 4 °C) and determined by Differential Pulse Polarography (DPP) analysis (Mouneyrac et al., 2002). The standard addition method was used for calibration with rabbit liver MT (Sigma Chemical Co., St. Louis, MO) in the absence of a marine bivalve MT standard.

For GST, CAT, SOD, TBARS and ChE, pooled soft tissues were homogenised at 4 °C to prevent enzyme or tissue degradation in TRIS buffer (TRIS 50 mM, NaCl 150 mM, DTT 1 mM, antiprotease mixture (Sigma P8340, diluted in 1/1000) adjusted to pH 7.4 in a 1:3 ratio (w:v) using a motor-driven glass-Teflon homogenizer at 500 rpm. The homogenates were then centrifuged for 25 min at 9000*g*. Supernatants were immediately frozen at −80 °C as 50 μL aliquots until biomarker analysis. An aliquot of the homogenate was centrifuged (9000*g* for 30 min at 4 °C) and the resulting supernatant was used directly in the enzyme assay. GST activity was determined spectrophotometrically at 340 nm ($\varepsilon = 9.6$ mM^{-1} cm^{-1}) by monitoring the formation of 1-glutathion-2,4-dinitrobenzene, resulting from the conjugation of the substrate, 1-chloro-2,4-dinitrobenzene (CDNB), with glutathione reduced form (GSH), as described by Habig et al. (1974). Results were expressed as nmoles of glutathione conjugate produced per min and per mg protein (nmoles min^{-1} mg^{-1} protein). CAT activity was estimated spectrophotometrically as the decrease in absorbance at 240 nm ($\varepsilon = 0.04$ mM^{-1} cm^{-1}) due to dismutation of hydrogen peroxide (H_2O_2) according to Claiborne (1985). Results were expressed as μmoles of H_2O_2 transformed per min and per mg protein (μmoles min^{-1} mg^{-1} protein). SOD activity was determined as the degree of inhibition of cytochrome C reduction by superoxide anion radicals generated by xanthine oxydase/xanthine reaction at 550 nm (McCord and Fridovich, 1969). Results were expressed as SOD Unit per mg protein (SOD Unit mg^{-1} protein). One unit of SOD activity was defined as the amount of sample producing 50% inhibition in 1 mL reaction system per mg protein. ChE activity was determined using the method of Ellman and Courtney (1961) adapted to a microplate reader by Galgani and Bocquene (1991). Results were expressed as nmoles of thiocholine produced per min and per mg protein (nmoles min^{-1} mg^{-1} protein). In the case of *H. diversicolor*, Scaps and Borot (2000) have characterized the presence of acetylcholinesterase whereas in *S. plana* it is not clear if only AChE or also pseudocholinesterases are able to hydrolise the substrate used. Thus in the all paper, we will use the abbreviation ChE for cholinesterases. Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS), quantified by reference to MDA absorbance ($\varepsilon = 1.56 \times 10^5$ M^{-1} cm^{-1}) at 530 nm (Halliwell and Gutteridge, 1985). Results were expressed as nmoles of TBARS produced per mg protein (nmoles of TBARS mg^{-1} protein).

For LDH determination, an aliquot of the homogenate prepared as described above for GST (but with individual specimens) was centrifuged at 3300*g* for 5 min at 4 °C. Measurement of LDH activity was realized with a microplate reader as described in Diamantino et al. (2001). Proteins were quantified in the supernatants according to Bradford (1976).

2.6. Metal quantification in animals

For bivalves, Cu was determined in the pellets and supernatants, the preparation of which has been described above for MTLP determination. Cu bioaccumulation was estimated in the whole soft tissues of worms (*H. diversicolor*, $n = 9$ per condition). Animal tissues were digested by heating (90 °C) with Aqua Regia (HCl/HNO_3:75/25) after preliminary tests showing that pure HNO_3 was not sufficient to insure a complete dissolution of Cu from CuO NP. Then, the quantification of Cu concentrations was performed by using Flame Atomic Absorption Spectrophotometry (FAAS, Varian SpectrAA800 spectrophotometer) with deuterium lamp background correction. Standard addition analyses were performed in an isomedium and added concentrations of Cu were 125 ng mL^{-1}, 250 ng mL^{-1}, 500 ng mL^{-1}, 1000 ng mL^{-1}. All labware were cleaned in 10% hydrochloric-acid bath for 24 h and rinsed three times with DIW before being used. The accuracy of

the analyses was checked by digesting certified material (Mussel tissue, National Institute of Standards and Technology). In the case of bivalves, the total concentration was recalculated by adding concentrations of Cu in pellet and supernatant.

2.7. Behavioural experiments

For burrowing tests, 20 bivalves were collected from the three different tanks ($n = 6$ or $n = 7$ taken randomly from each tank) corresponding to each experimental condition. For feeding tests, all the bivalves were involved ($n = 36$ per condition distributed in three different tanks). Both burrowing and feeding tests were carried out with 20 ragworms per condition. At the end of the behavioural tests, animals were returned in their experimental medium of origin. This procedure is acceptable since Burlinson and Lawrence (2007) have shown that burrowing organisms were not affected by consecutive behavioural assays.

2.7.1. Burrowing tests

Bivalves (*S. plana*) and worms (*H. diversicolor*) were submitted to burrowing tests as described by Bonnard et al. (2009), after being previously exposed in the laboratory for 4 d to seawater only (controls), CuO NPs or dissolved Cu as described in exposure protocol sub-section (see above). For bivalves (*S. plana*), burrowing experiments were carried out in plastic containers filled with 3 cm of natural sediment (collected from sampling site) and topped up with 2 L of seawater. Natural sediment was homogenised by hand 1 d before experimentation. Burrowing behaviour was studied by placing individuals on the surface of the sediment and observing the number which had burrowed at frequent intervals; every 5 min in the 1st hour, every 10 min in the 2nd hour, every 20 min in the 3rd and 4th hour, then every hour until 6 or 7 h of test. Twenty individuals of *H. diversicolor* were tested for each studied condition. Briefly, we used plastic clean containers of 100 mL filled with 5 cm of wet sediment from the site of origin. Twenty worms were placed individually on the sediment and their positions were recorded every two min during 30 min.

2.7.2. Feeding rate

The feeding rate was estimated for bivalves (*S. plana*) and worms (*H. diversicolor*) previously exposed for 11 or 7 d respectively, to seawater only (Controls), CuO NPs or soluble Cu. The feeding rate of *S. plana* was quantified according to the methodology described by Worrall and Widdows (1983) and adapted to our conditions. Algae *Tetraselmis suecica* supplied by IFREMER were used as food at a concentration of 10 000 cell mL^{-1} in each tank. The concentration of algae not ingested by bivalves was measured after 1 h. The feeding rate of *H. diversicolor* was quantified according to the methodology described by Moreira et al. (2005). Twenty worms were fed 100 *Artemia salina* larvae into their individual plastic beakers containing 50 mL of seawater. They were left undisturbed for 1 h, then the remaining larvae were collected and

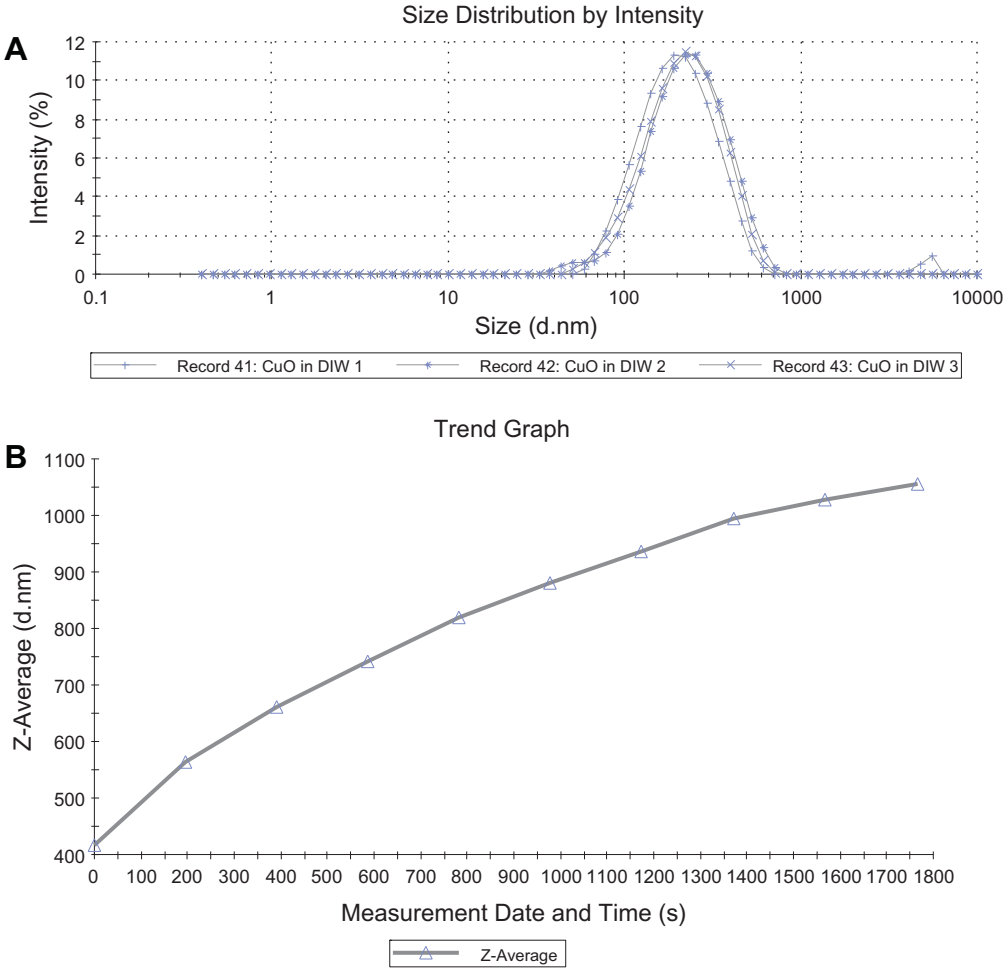

Fig. 1. Size distribution (A) of CuO nanoparticles in DIW and evolution of the size of CuO nanoparticles when a drop of sea water ($t = 2$ d) is incorporated in the DIW suspension (B).

counted and results were expressed as the number of larvae ingested per hour per individual.

2.8. Statistical analysis

The burrowing kinetic curves were ln-transformed in order to linearize these data and then, they were compared by using analysis of covariance (ANCOVA) between regression coefficients of the least-square best-fit regression lines. Otherwise, results are presented as mean ± SD. Significant differences were established by using one-way analysis of variance (ANOVA) or non-parametric Mann and Whitney comparison tests when variances of groups were different. Level of significance was established at $p \leqslant 0.05$. Statistical analyses were performed by using Xlstat pro 7.5.

3. Results

3.1. Fate of CuO nanoparticles in the experimental medium

Size distribution of CuO NP in DIW ranged from 40 nm to 500 nm with an average of 197 nm (Fig. 1A) and the prepared suspension was stable for approximately 1 month. When diluted in sea water (at both $t = 0$ and $t = 2$ d where animal stayed during 2 d), the NPs aggregate/agglomerate and the sample is not suitable for DLS measurements. Fig. 1B shows how rapidly the size of the particles increases when 5 μL of sea water is added in the DIW suspension. Zeta potential measurements show that the particles are relatively highly positively charged (26.3 mV) in DIW, which corroborates the stability of the suspension, while the NPs appear slightly negatively charged in seawater collected at $t = 0$ and $t = 2$ (−8.69 and −7.72 mV respectively), an indication of poor stability.

3.2. Cu in the exposure medium

Fig. 2 shows the mass of copper accumulated by DGT units during the 16 d of laboratory exposure to seawater only (Controls), dissolved Cu or CuO NPs. The results obtained for DGT exposed to CuO nanoparticles, at a nominal concentration of 10 μg of Cu L^{-1}, demonstrated that the masses of copper accumulated were similar to those accumulated by DGT units deployed in control tanks and significantly lower than those obtained with DGT units exposed to soluble copper (respectively on day 16: 0.338 ± 0.012 μg; 0.383 ± 0.055 μg, 1.017 ± 0.033 μg). This result indicates that no detectable liberation of labile copper from NPs occurred in the medium during the time of experiment.

3.3. Cu bioaccumulation

Cu concentrations in the whole soft tissues of *S. plana*, exposed in the laboratory during 16 d and *H. diversicolor* exposed during 7 d to seawater only (Controls), CuO NPs (10 μg Cu L^{-1}) and soluble Cu (10 μg Cu L^{-1} in 1 M of HNO_3) are illustrated in Table 1. Bivalves (*S. plana*) and worms (*H. diversicolor*) exposed to both forms of copper (CuO NPs and soluble Cu) showed significantly higher concentrations compared to controls. In addition for both species, no significant differences were observed depending on the form of Cu in the exposure medium.

3.4. Biomarkers

Measurements of biomarkers of defences and damages in bivalves (*S. plana*) and worms (*H. diversicolor*) are illustrated in Table 2. In both species exposed to CuO NPs, catalase and GST activities increased significantly compared to controls. In *H. diversicolor*, catalase activity also increased significantly after soluble Cu exposure treatments. In bivalves, SOD activity was significantly higher in animals exposed to CuO NP compared to controls or animals exposed to soluble Cu. MTLP concentrations increased in bivalves exposed to both forms of copper (soluble Cu, CuO NPs), but significantly only for soluble Cu. For both species, no damages were observed according to the results of TBARS, LDH and ChE.

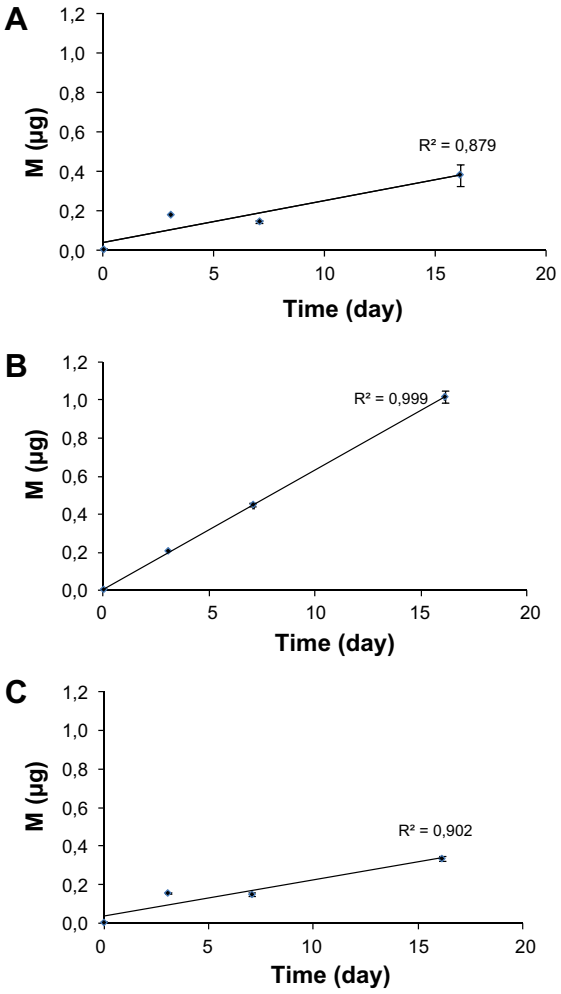

Fig. 2. Mean mass (and standard deviation) of copper accumulated (μg) by DGT units ($n = 8$) during the time of exposure. (A) Control; (B) Soluble Cu; (C) CuO NP.

Table 1
Mean (ng g^{-1} wet weight) copper concentrations (and SD between brackets) in *Scrobicularia plana* and in *Nereis diversicolor* (whole soft tissues) exposed in the laboratory to Cu (10 μg L^{-1}) either in the form of CuO NP (CuO NP), soluble Cu and seawater only (Controls). Concentrations with different superscripts differed significantly at the 95% level in each species.

	S. plana	*N. diversicolor*
Controls	3634 (1915)^{a}	1006 (132)^{c}
Soluble Cu	5998 (3023)^{b}	1431 (583)^{d}
CuO NP	7495 (2379)^{b}	1570 (302)^{d}

3.5. Burrowing behaviour

The burrowing behaviour of *S. plana* and *H. diversicolor* (previously exposed for 4 d to soluble Cu, CuO NPs as well as controls) in sediment collected from their site of origin is shown in Fig. 3A and B. Slopes and regression coefficients of the best-fit regression

Table 2
Means (and SD between brackets) of biomarkers quantified in *Nereis diversicolor* after 7 d and *Scrobicularia plana* after 16 d of exposure treatments (Controls, soluble Cu, CuO NP). For both species, biomarker levels with different superscripts differed significantly at the 95%.

Biomarkers		*Scrobicularia plana*			*Nereis diversicolor*		
		Controls ($n = 5$)	Soluble Cu (10 µg L^{-1}) ($n = 5$)	CuO NP (10 µg L^{-1}) ($n = 5$)	Controls ($n = 5$)	Soluble Cu (10 µg L^{-1}) ($n = 5$)	CuO NP (10 µg L^{-1}) ($n = 5$)
Defences	CAT (µmol min^{-1} mg^{-1} protein)	66.50 (16.59)^{a}	80.98 (3.53)^{a}	119.35 (17.70)^{b}	53.27 (2.46)^{a}	78.05 (9.35)^{b}	96.51 (13.45)^{c}
	GST (nmol min^{-1} mg^{-1} protein)	260.87 (43.68)^{a}	311.40 (31.15)^{a}	423.18 (36.26)^{b}	81.19 (27.54)^{a}	94.60 (31.84)^{ab}	130.99 (11.81)^{b}
	SOD (U SOD mg^{-1} protein)	6.40 (1.25)^{a}	6.47 (0.82)^{a}	10.23 (2.67)^{b}	75.45 (9.5)	73.76 (28.00)	72.45 (3.8)
	MTLP (µg g^{-1} tissues)	876.12 (182.23)^{a}	1060.02 (211.85)^{b}	1016.82 (130.17)^{ab}			
	LDH (nmol min^{-1} mg^{-1} protein)	20.57 (5.49)	18.97 (7.14)	23.06 (10.72)	552.03 (89.91)	514.02 (96.43)	569.50 (208.90)
Damages	TBARS (nmol MDA mg^{-1} protein)	0.52 (0.11)	0.50 (0.05)	0.53 (0.23)	0.76 (0.20)	0.77 (0.12)	0.90 (0.014)
	AChE (nmol min^{-1} mg^{-1} protein)	22.36 (6.24)	26.37 (9.28)	28.49 (10.71)	71.33 (14.23)	70.26 (17.69)	70.14 (8.61)

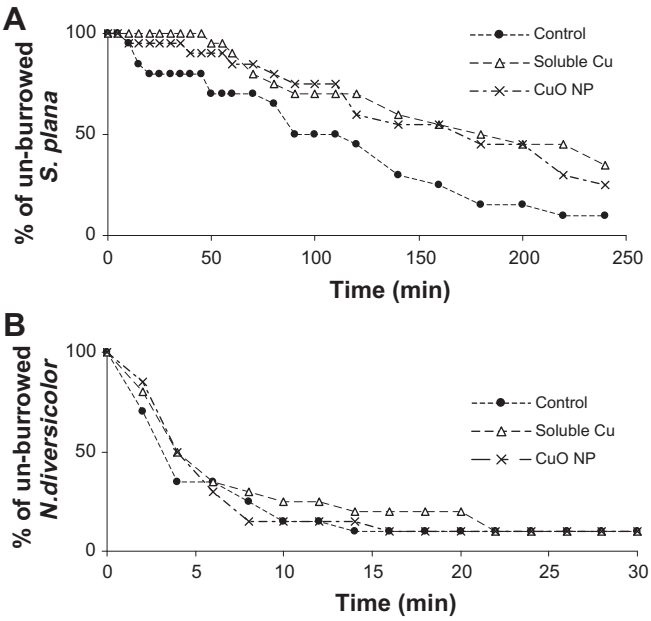

Fig. 3. Percentage of unburrowed clams (A) *Scrobicularia plana* ($n = 20$) and ragworms (B) *Hediste diversicolor* ($n = 20$) at different times (min). Animals were previously exposed to the different experimental conditions: sea water only (control ●), soluble copper Δ, CuO nanoparticles ×; and allowed to burrow in their sediment of origin (Bay of Bourgneuf).

lines obtained after ln transformation of the raw data are shown in Table 3. In *S. plana*, the burrowing behaviour was significantly impaired in bivalves exposed to CuO NP or to soluble form of Cu compared to controls. In the case of *H. diversicolor*, only the exposure to soluble Cu led to a significant decrease of burrowing kinetics compared to controls and CuO NP exposure.

3.6. Feeding rate

Feeding rates of *S. plana* and *H. diversicolor* were exposed (controls, soluble Cu, CuO NP) for 11 d or 7 d respectively. During the first hour of feeding, bivalves (*S. plana*) in control medium showed the highest feeding rates (6051 ± 1003 cells h^{-1}) while feeding rates were significantly impaired only for specimens from CuO NP exposure medium (3680 ± 1294 cells h^{-1}).

Concerning *H. diversicolor* feeding rates were not affected by experimental conditions (not shown).

4. Discussion

4.1. Exposure

The environmental fate and ecotoxicity of engineered NPs may be influenced by a number of properties, including particle size and

Table 3
Slopes and determination coefficients of the best-fit regression lines obtained after ln transformation of the raw data shown in Fig. 3. Slopes with different superscripts differed significantly at the 95% level.

Experimental conditions	Animals	Slope	Coefficient R^2
Control	*S. plana*	−0.0096^{a}	0.9604
Soluble Cu	*S. plana*	−0.0043^{b}	0.9664
CuO Nanoparticles	*S. plana*	−0.0051^{b}	0.9318
Control	*N. diversicolor*	−0.1433^{a}	0.9478
Soluble Cu	*N. diversicolor*	−0.0734^{b}	0.8545
CuO Nanoparticles	*N. diversicolor*	−0.132^{a}	0.8299

size distribution, state of aggregation, charge and solubility. Following release to the environmental systems, most engineered NPs are able to aggregate to some degree. The degree and kinetics of aggregation and the size range of the aggregates is dependent on the characteristics of the particles, the characteristics of the environmental system, and the concentration of the nanoparticles. As aggregation is likely to affect toxicity, characterization of NP in experimental media is essential for nanoecotoxicology (Tiede et al., 2009). In the present study, the particle size of CuO NP specified by the manufacturer ranged from 10 to 100 nm. However, actual characterization of CuO NP in DIW showed that particle size ranged from 40 nm to 500 nm with an average of 197 nm. In seawater, the CuO NP highly aggregated/agglomerated and the hydrodynamic size (or Z-average) increased rapidly to values of around 1000 nm within the duration of the test. According to Fabrega et al. (2011) aggregation enhances the rate at which NPs sink to the bottom, where their ingestion by animals that feed on sediments is possible. This mechanism is likely to occur in the case of *S. plana* which is "primarily a deposit-feeder [which] obtains only some of its food by filtering suspended matter from the sea" Hughes (1969). *H. diversicolor*, as an opportunistic species, is considered able to fulfil its energy needs using different kinds of diet (Olivier et al., 1995). However, the aggregation/agglomeration of most NPs detected by DLS may not record the existence of some free NPs in the media, as previously expressed by Griffitt et al. (2008).

In the case of metal-containing NPs such as CuO NPs, the release of metal ions and their speciation may also be a key factor in their (eco)toxicity. The presence of organisms can affect particle dissolution in exposure medium (Griffitt et al., 2008). Thus, in this work, we studied the putative dissolution of Cu from CuO NP in real exposure (in the presence of animals) to estimate the contribution of dissolved Cu ions to overall cytotoxic effects. Solubility of copper oxide in water has been extensively studied under various pH and temperature conditions (Palmer et al., 2004). Models of bioavailability and of toxicity of copper ions have been proposed (Luoma and Rainbow, 2008). Since Zhang and Davison (1999) have described the DGT method, this has been widely used in environmental studies dealing with metal speciation in water (Pesavento et al., 2009). In the conditions of our experiment, DGT results indicated that no measurable release of labile Cu from CuO NPs occurred during the time of experiment (16 d). Griffitt et al. (2008) showed similar results, with less than 1% by mass of the original dose present in dissolved form after 48 h for copper in a freshwater medium. In contrast, Blinova et al. (2010) found, also in freshwater, higher copper dissolution (12% of copper) from CuO NP using *Escherichia coli* Cu-sensor. This present work demonstrates the interest of using DGT techniques as a proxy to estimate the bioavailability of metals released from NP under soluble form in the aquatic environment.

4.2. Cu bioaccumulation

As documented earlier, both bivalves and worms exposed to soluble Cu in the experimental medium have incorporated Cu. Due to the limited period of exposure, the concentrations reached at the end of the experiment were orders of magnitude lower than those uncountered in the most polluted estuaries (Bryan et al., 1980). Concentrations as high as 752 $\mu g\, g^{-1}$ dw (about 150 $\mu g\, g^{-1}$ ww) were determined in *S. plana* from the Erme estuary, UK and 1430 $\mu g\, g^{-1}$ dw (about 286 $\mu g\, g^{-1}$ ww) in *Nereis* (*Hediste*) *diversicolor* from Restronguet Creek, UK. More interestingly, the concentrations reached in each species exposed to Cu as nanoparticles were identical to those due to soluble Cu, despite DGT results did not indicate any measurable release of labile Cu from CuO NPs occurring during the time of experiment. However, NP can also be taken into the gut providing additional route for metal bioavailability. In a recent study, Galloway et al. (2010) using transmission electronic microscopy (TEM), observed that TiO_2 NPs were localized in the gut lumen in the marine polychaete *Arenicola marina*. Because we have seen that CuO NP was remarkably stable since pure HNO_3 was insufficient to solubilized them (see Section 2.6) it is less probable that they may be partially solubilized in the gut due to the moderate pH value in the gut of bivalves (pH: 4–5.2) and worms (pH: 5.5–6).

4.3. Ecotoxicity effects

The fate and ecotoxicity of particles of nanometric size in endobenthic organisms such as the bivalve mollusc *S. plana* and the ragworm *H. diversicolor* is very poorly known. Questions appeared since recent studies showed that NP can be more toxic than their bulk form (Blinova et al., 2010; Heinlaan et al., 2008) or micrometric particles (Karlsson et al., 2009). It was previously shown that the toxicity of bulk and CuO NPs was due to the solubilized bioavailable fraction, most likely Cu^{2+} ions (Heinlaan et al., 2008). Since no additional labile Cu in seawater exposure media (compared to controls) was registered during the time of experiment (16 d), we have to investigate about the specific toxicity from nanoparticulate copper.

Induction of MT, a cystein-rich protein that binds metals and thus contributes to metal detoxification, has been reported in various aquatic species under *in situ* or laboratory metals exposure (Amiard et al., 2006). In addition, MTLP may be involved in the defence against oxidative stress (Viarengo et al., 1999). Several studies reported that nanoparticles contribute to oxidative stress (Unfried et al., 2007). Independently of any nanotoxicity, the Cu toxicity may be caused at least partly by the generation of reactive-oxygen species (Sun et al., 2009 and literature cited therein). In this work, an increase of MTLP was observed in bivalves exposed to Cu either as CuO NPs or soluble Cu. CAT, GST and SOD have been classified as antioxidant systems of defence in various aquatic species such as bivalves (Almeida et al., 2007). CAT and GST activities increased significantly in both species (*S. plana*, *H. diversicolor*) and SOD in *S. plana* exposed to CuO NP, suggesting an oxidative stress endured by animals. In *H. diversicolor* from a contaminated estuary, an enhancement of LDH activity (up to 1.5 fold) was interpretated as an increased rate of organisms' anaerobic metabolism, suggesting a rapid need of additional energy to ameliorate chemical stress (Moreira et al., 2006). In the present study, no changes in LDH activity were observed in both species in any cases of experimental exposure. However, during progressive or prolonged anoxia, many marine invertebrates rely on different metabolic pathways (e.g. the glucose-succinate pathway) to maintain a production of metabolic energy (for a review see Livingstone, 1991). For example, succinate is a sensitive indicator of anoxia in bivalves (Oeschger and Pedersen, 1994) or in marine polychaetes (Völkel and Grieshaber, 1992).

Concerning biomarkers of damages, no alteration of TBARS levels, and ChE activities were depicted in any cases and in both species, under the experimental conditions. It may be suggested that the antioxidant defences could play their role by preventing oxidative damage. However, defence mechanisms were probably not sufficient to prevent behavioural impairments. Burrowing kinetics were significantly impaired in *S. plana* exposed to both forms of Cu (CuO NP or soluble Cu) compared to controls whereas for feeding rate only in the case of CuO NP exposure. In the case of *H. diversicolor*, only the exposure to soluble Cu led to a significant decrease of the burrowing kinetic of worms and feeding rate seemed not to be affected by experimental conditions. In a similar way, Wallace et al. (2000) showed that saturation of Cd-MT in grass shrimp fed cadmium-contaminated preys was associated with decreased the prey capture.

In invertebrates, tolerance to environmental contaminants is based upon a large range of chemical handling strategies. So it is not surprising that responses differ between a bivalve and a polychaete. In the case of toxic metals but also essential metals in large excess, a first line of defence is based on MT as a detoxificatory ligand. Previous works have well-established that the way MT plays a protective role is different between these taxa. The concentration of this metalloprotein is generally increased in bivalves exposed to metal pollution, providing directly a detoxificatory ligand (Amiard et al., 2006) whereas in certain polychaete species, it is MT turnover which is substantially increased in response to metal exposure, probably allowing the transfer from cytosol to detoxificatory granules (Ng et al., 2008). In *H. diversicolor* originating from highly Cu contaminated site, Cu granules were observed as a major sink for bioaccumulated Cu (Mouneyrac et al., 2003).

Links between behavioural impairment and AChE inhibition is well-documented for aquatic biota (Amiard-Triquet, 2009). In the present study, behavioural impairments were not accompanied by an inhibition of ChE activity, but metabolical or physiological disturbances due to the "cost of tolerance" could be the cause of these impairments (Bonnard et al., 2009).

5. Conclusions

In conclusion, the Cu concentrations used in this study either in the form of CuO NPs or soluble Cu were able to induce biological effects. It must be highlighted that the chosen soluble Cu concentrations of 10 $\mu g\ L^{-1}$ may be encountered in polluted environments (since there is no available measured or predicted data on CuO NP concentrations). Several biomarkers of defence are activated in the presence of CuO NPs, namely GST, CAT and SOD in *S. plana* and CAT and GST in *Hediste diversicolor*. These defences may be relatively efficient since no significant effects were shown considering individually biochemical markers of neurotoxicity (ChE) or oxidative damage (TBARS). However, behavioural impairments were observed in the bivalve (*S. plana*), considering both the burrowing and feeding behaviours. For a number of biomarkers, it must be noted that biological responses are significantly more important in the presence of CuO NPs than in the presence of soluble Cu. Thus, these results suggest a specific nanoparticle effect. Finally, the use of both marine invertebrates (*S. plana*, *H. diversicolor)* and the set of biomarkers chosen in this work seem suitable to conduct ecotoxicity studies with ENPs. With a view to risk assessment it is necessary to improve the evaluation of exposure and the subsequent bioaccumulation. Future works (TEM) are in progress to localize cellular targets of CuO NPs in both species.

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