Pradhan A, Seena S, Pascoal C, Cássio F (2012) Copper oxide nanoparticles can induce toxicity to the freshwater shredder *Allogamus ligonifer*. *Chemosphere* 89: 1142-1150
Submitted version

Copper oxide nanoparticles can induce toxicity to the freshwater shredder *Allogamus ligonifer* via aqueous or dietary exposure

Arunava Pradhan, Sahadevan Seena, Cláudia Pascoal and Fernanda Cássio

Centre of Molecular and Environmental Biology (CBMA), Department of Biology,

University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal

For correspondence:

Fernanda Cássio

E-mail fcassio@bio.uminho.pt

Tel. +351253604045

Fax. +351253678980

Pradhan A, Seena S, Pascoal C, Cássio F (2012) Copper oxide nanoparticles can induce toxicity to the freshwater shredder *Allogamus ligonifer*. *Chemosphere* 89: 1142-1150
Submitted version

Abstract

The increased commercialisation of nano metal-based products augments the possibility of their terminal deposition into aquatic ecosystems, which, in turn, may pose risks to aquatic biota and associated ecological functions. Freshwater invertebrate shredders mostly use microbially-colonized plant litter as food resource and play an important role in aquatic detritus food webs by transferring nutrients and energy to higher trophic levels. We assessed effects of nano CuO on the shredder *Allogamus ligonifer* (Trichoptera, Limnephilidae) by determining the concentration that induced 50% of death (LC_{50}) and the effects of sublethal concentrations on the feeding behaviour and growth of the shredder. In sublethal toxicity tests, we examined the effects of nanoparticles i) via contaminated water, by exposing the shredder to stream water supplemented with nano CuO (0, 25 and 75 mg L^{-1}) and microbially-colonized leaves, and ii) via contaminated food, by exposing the shredders to stream water (without nano CuO) and microbially-colonized leaves pre-exposed to nano CuO (0, 25 and 75 mg L^{-1}). Results from acute lethal tests showed that the 96 h LC_{50} of nano CuO was very high (569 mg L^{-1}). A significant inhibition in leaf consumption rate (up to 47%) and invertebrate growth rate (up to 46%) was observed when shredders were exposed to the highest sublethal concentration of nano CuO through either stream water or diet. The exposure to increased nano CuO concentration via water or diet led to higher accumulation of copper in the larval body. Leached ionic copper from the nano CuO adsorbed to or accumulated in the shredder seemed to influence the feeding behaviour and growth of the shredder.

Keywords: Nano CuO, freshwater shredder, lethal effect, sublethal effects, aqueous and dietary exposure, feeding behaviour

1. Introduction

Nanoecotoxicology research is currently in the limelight due to high propagation of nanotechnology-based industries and nanomaterial-based products (Aitken et al., 2006; Colvin, 2003; Navarro et al., 2008). The extensive use of the engineered nanomaterials may increase the possibilities of their leaching and deposition into aquatic reservoirs (e.g. Kaegi et al., 2008). Therefore, it is essential to understand the risks associated with tailored nanoparticles in aquatic ecosystems (MacCormack and Goss, 2008; Moore, 2006). Metal oxide nanoparticles are among the most frequently used nanomaterials having a broad range of applications, like in sunscreens and cosmetics (Nel et al., 2006), antimicrobial paints (Hochmannova and Vytrasova, 2010), textiles (Becheri et al., 2008; Kathirvelu et al., 2009), electrospray disinfectants (Wang et al., 2010), drug delivery and gene therapy (Jin and Ye, 2007). Over the last decade, several studies have reported that metal oxide nanoparticles are potentially toxic (see Reijnders, 2006 and Gajjar et al., 2009), but few attempts have been made to assess the ecotoxicity of nano metal oxides in aquatic systems (Blaise et al., 2008; Lee et al., 2009; Miller et al., 2010; Pradhan et al., 2011). Most studies were performed with the nano metal oxides enlisted in the OECD guidance manual (OECD, 2010), like nano titanium dioxide, nano zinc oxide, nano aluminium oxide and nano cerium dioxide (Lovern et al., 2007; Van Hoecke et al., 2009; Zhu et al., 2008). However, the OECD guidance manual stresses that the enlisted nanoparticles have to be considered as a "snapshot in time" and those not included in the list can be of importance in the future (OECD, 2010).

Although nano copper oxide (CuO) is not in the OECD list, it is one of the commercially manufactured metal oxide nanoparticles with wide range of applications (Carnes and Klabunde, 2003; Dutta et al., 2003; Ren et al., 2009; Zhang et al., 2008) and, therefore, its potential toxicity should not be ignored (Blinova et al., 2010; Buffet et al., 2011; Saison et al., 2010). The toxicity of the nano-sized metals in aquatic systems can be

questionable (Sharma, 2009) as they have different properties than their bulk or ionic forms (Christian et al., 2008). Karlsson et al. (2009) showed in human cell lines that nanoparticles of CuO could be more toxic than the bulk micrometer particles. However, the toxicity of nano CuO and other metal oxide nanoparticles to yeasts (Kasemets et al., 2009) and other organisms that are crucial in aquatic food webs, like microalgae (Aruoja et al., 2009), protozoa (Mortimer et al., 2010), bacteria and crustaceans (Heinlaan et al., 2008), was attributed to the leached ionic form of the metal.

In freshwaters, invertebrate shredders decompose plant litter from the riparian vegetation and play a key role in detritus food web by transferring energy from plant litter to higher trophic levels (Graça and Canhoto, 2006). They prefer to feed on litter colonized by aquatic microbes, predominantly fungi, which activity increases the food quality and palatability to shredders (Suberkropp et al., 1983). Invertebrates are important test organisms in ecotoxicological studies as they are abundant, distributed worldwide, have short life span with high reproduction rates, and are sensitive to contaminants and toxicants including ionic metals (e.g., De Schamphelaere et al., 2004; Gerhardt et al., 2004) and nano metal oxides (Cattaneo et al., 2009; Buffet et al., 2011; Galloway et al., 2010). Moreover, ecotoxicological tests using freshwater invertebrate shredders are fast, cost-effective and easy to perform as invertebrates adapt quickly to the laboratory conditions.

Most studies reporting lethal toxicity of ionic copper, nano sized copper and its oxides on aquatic invertebrates are based on the assumption that metal toxicity to aquatic biota occurs through waterborne exposure (Griffitt et al., 2008; Heinlaan et al., 2008, 2011). Indeed, very few studies have shown that ionic copper can have sublethal toxic impacts to aquatic invertebrates through dietary exposure (De Schamphelaere et al., 2007; Hatakeyama, 1989), but according to our knowledge none of the studies reported the dietary effects of nano copper oxide on stream invertebrates.

The aim of this study was to investigate the potential impacts of nano CuO on *Allogamus ligonifer*, a common invertebrate shredder in Southwest European streams that prefers high quality stream water (Bonada et al., 2008). We hypothesised that nano CuO can pose toxicity to the invertebrate shredder through both aqueous and dietary exposure, and impacts would be partially attributed to the bioavailable ionic copper leached from nano CuO. We assessed the acute lethal effect of nano CuO through aqueous exposure by monitoring the mortality of *A. ligonifer* up to 96 h. The sublethal toxicity through aqueous (stream water) or dietary (microbially-colonized leaves) exposure was examined by assessing the feeding behaviour and growth rate of the invertebrate shredder. Total copper and ionic copper in the stream water, leaves, body and case of the shredder was determined in an attempt to discriminate the contribution of nano and leached ionic copper to toxicity.

2. Material and Methods

2.1 Microbial colonization of leaves in the stream

Leaves of *Alnus glutinosa* (L.) Gaertn. (alder) were collected from a single tree in autumn and air dried at room temperature. The leaves were soaked in deionised water, cut into 12 mm-diameter disks, and placed into fine-mesh bags (15 × 15 cm, 0.5-mm mesh size to prevent invertebrate colonization). In Spring 2010, leaf bags were immersed in the Maceira Stream (N 41°45'58.79", W 8°08'49.39", altitude 867 m, Cávado River basin, Northwest Portugal) to allow microbial colonization. After 10 days, leaf bags were retrieved and leaf disks from each replicate bag were rinsed with deionised water and used for the feeding experiment. Further information on the Maceira Stream can be found elsewhere (Duarte et al., 2009; Pradhan et al., 2011).

2.2 Collection of invertebrates and acclimation to the laboratory

Early-stage larvae of the caddisfly *Allogamus ligonifer* (McLachlan, 1876) with similar size were collected in the upper reach of the Cávado River in Spring 2010 and transported to the laboratory in plastic containers with stream water and sand. This stream detritivore that belongs to Limnephilidae occurs in Southwest Europe (Bonada et al., 2008) and is common in low-order streams of North Portugal (Varandas and Cortes, 2010). Further information on the Cávado River can be found elsewhere (Pascoal et al., 2001). In the laboratory, animals were placed in an aquarium with filtered (MN GF-3 filter paper, Macherey-Nagel, Germany) and sterile stream water and sand (121°C, 20 min) under aeration, at 14°C with a 12 h light : 12 h dark photoperiod, and were allowed to feed on alder leaves for 2 weeks before the experiment.

2.3 Preparation and characterization of nano copper oxide suspension

The stock suspension of nano copper oxide (CuO nanopowder <50 nm, 99.5%, Sigma-Aldrich, St. Louis, MO) was prepared in sterile stream water by sonication at 42 kHz in a sonication bath (Branson 2510, Danbury, CT, USA) for 30 min in dark before use (Heinlaan et al., 2008). The stream water had silica 9.6 ± 2 mg L^{-1}, Na^+ 4.1 ± 0.4 mg L^{-1}, Ca^{2+} 1.3 ± 0.3 mg L^{-1}, K^+ 0.6 ± 0.1 mg L^{-1}, HCO_3^- 8.0 ± 0.8 mg L^{-1}, Cl^- 4.2 ± 0.4 mg L^{-1}, and SO_4^- 1.0 ± 0.2 mg L^{-1}. The pH of stock suspension was adjusted to 5.8 ± 0.2. The stock suspension was examined with UV-visible spectrophotometry (UV – 1700 PharmaSpec, Shimadzu, Kyoto, Japan) followed by scanning electron microscopy (SEM, Leica Cambridge S 360, Cambridge, UK) coupled to an energy dispersive X-ray microanalysis setup (EDX, 15 KeV) as described by Pradhan et al. (2011). The particle size of nano CuO, measured by SEM, ranged between 30 and 50 nm that complies with the manufacturer specification (not shown).

The size distribution was also monitored by dynamic light scattering (DLS) using a zetasizer (Malvern, Zetasizer Nano ZS) to check agglomeration of nano CuO in the stock

suspension. DLS data showed that the size distribution of nano CuO ranged from 120 to 340 nm with an average size of 202 nm and poly-dispersive index (PdI) of 0.186 (Fig 1). The stability was confirmed up to 3 weeks. The increased average particle size observed by DLS compared to the measured particle size by SEM indicated agglomeration of nano CuO in the stream water which agrees with previous observations in deionized water (Buffet et al., 2011) and liquid culture medium (Karlsson et al., 2009).

2.4. Acute lethality tests

Acute lethality tests were performed to evaluate the sensitivity of the invertebrate to nano CuO and to establish a range of sublethal concentrations to be used in the feeding experiments (see section *2.5*). Invertebrate shredders were starved for 24 h and placed in 150 mL flasks containing 100 mL of nano CuO suspensions (5 animals per flask, 3 replicates per treatment). The animals were exposed to 0, 50, 100, 250, 500 and 1000 mg L^{-1} nominal concentrations of nano CuO prepared in sterilized stream water. The flasks were aerated with constant air flow and incubated for 96 h at 14°C, under a 12 h light : 12 h dark photoperiod. The invertebrates were not fed during the exposure period. In each 24 h, the animals that did not show any movement after mechanical stimulation were considered dead.

2.5 Invertebrate feeding experiments

To determine effects of nano CuO on the feeding behaviour of the invertebrate shredder, one premeasured early-stage larvae of the invertebrate species *A. ligonifer* was allocated to each of 150 mL flask containing 10 leaf disks and 100 mL sterile stream water. To assess the effects of the nanoparticles via water, stream water was supplemented with nano CuO at 25 mg L^{-1} or 75 mg L^{-1} and microbially-colonized leaf disks unexposed to nano CuO. To test the effects of the nanoparticles via diet, flasks were supplemented with stream

water (without nano CuO) and microbially-colonized leaf disks pre-exposed (5 days) to 25 mg L^{-1} or 75 mg L^{-1} of nano CuO. Additional flasks served as control and were provided with sterile stream water and microbially-colonized unexposed leaf disks. A total of 75 flasks were used (15 replicates).

For determining the contribution of microorganisms to leaf litter decomposition, an equal number of unexposed or pre-exposed leaf disks to nano CuO was enclosed in 0.5 mm fine mesh bag (to prevent the access of invertebrates) and placed in each replicate flask of the respective treatment. All flasks were aerated with constant air flow and incubated at 14°C, under a 12 h light : 12 h dark photoperiod. The experiment was continued for 10 days until >50% of leaf disks were decomposed in the control flasks. The stream water with or without nano CuO was renewed every 5 days to minimise the interference of released fine particles or excreted compounds with nanoparticles or invertebrates.

2.6 Leaf mass loss

To determine leaf mass loss, leaf disks from each replicate were freeze-dried (Christ alpha 2–4, B. Braun, Germany) and weighed to the nearest 0.001 mg, before and after microbial colonization in the stream, and before and after the feeding experiment.

2.7 Leaf consumption by the invertebrate and microbes

Dry mass (DM, mg) of leaves consumed by the invertebrate (L_e) was determined as $(L_i - L_f) - (L_i \times (C_i - C_f)/C_i)$, where L_i and L_f are the initial and final dry mass (mg) of leaves exposed to the invertebrates, respectively, and C_i and C_f are the initial and final dry mass (mg) of control leaves (inaccessible to invertebrate), respectively. Microbial leaf decomposition rate was determined by $(C_i - C_f)/t$ where t is time (t=10 days). Leaf consumption rate by the invertebrate was calculated as $L_e/(I_f \times t)$, where I_f is the invertebrate

dry mass (mg) at time t (day 10), and results were expressed as mg leaf DM mg^{-1} animal DM day^{-1} (Ferreira et al., 2010). Total consumption rate was determined as $((C_i - C_f) + L_e)/t$ and expressed as mg leaf DM mg^{-1} $microcosm^{-1}$ day^{-1}.

2.8 Invertebrate growth rate

Growth rate of invertebrates (µg animal DM mg^{-1} animal DM day^{-1}) was determined as $I_e/(I_f \times t)$, where I_e is the dry mass (DM, µg) gained by the invertebrate during the elapsed time (t=10 days). The I_e was calculated by the difference between final (day 10) and initial dry mass (µg), and I_f is the final dry mass (mg) of the animal at time t (Ferreira et al., 2010). For determining initial dry mass of invertebrates, the diameter of the case opening of each individual was measured under a stereoscopic microscope at 16× before the feeding experiment, and the individual dry mass was estimated according to the regression model DM = $0.0069 \times CO - 0.0194$ ($r^2 = 0.72$, $P < 0.001$, $n = 37$), where DM is dry mass (g) and CO is case opening (mm).

2.9 Sample preparation and metal analysis

To determine total copper (suspended nano and ionic forms) and ionic copper (Cu^{2+}) in water, equal volume of water samples of all replicate flasks were mixed, and a fraction of 25 mL was ultra-centrifuged at 75,600 *g* for 60 min (Beckman Avanti J-25I, USA). The supernatant was consecutively filtered through two different size polycarbonate membranes (0.2 and 0.05 µm pore size, Millipore, Billerica, MA), and a mixed cellulose ester membrane (0.025 µm pore size, Millipore). The filtrate was employed to determine Cu^{2+} content. A separate fraction of 25 mL of water sample was treated with analytical grade concentrated HCl (5 mL) for quantification of total copper.

223 At the end of the feeding experiment, the Cu^{2+} leached from adsorbed or accumulated
224 nano CuO to leaves and to case and body of *A. ligonifer* was determined. For that, the freeze-
225 dried (Christ alpha 2–4, B. Braun, Germany) samples were revived in 25 mL ultrapure (Milli
226 Q) water for 60 min to allow the leaching of Cu^{2+}. Samples were ultra-centrifuged and
227 filtered as described above before Cu^{2+} quantification. To determine the adsorbed nano
228 copper, all the pellets from ultra-centrifugation and residues from filtration of each sample
229 were pooled and soaked in 25 mL of 5% HCl at 60°C; the solution was filtered through a
230 polycarbonate membrane filter of 0.2 μm pore size and collected for analysis. The remaining
231 residue was mineralized in the furnace at 550°C (16 h for leaves, 20 h for larval case and 10 h
232 for larval body) followed by digestion with HCl (1 mL) to determine the total accumulated
233 copper. The digested solutions were washed with 25 mL of 5% HCl, filtered through a
234 polycarbonate membrane filter of 0.2 μm pore size and used for determining bio-accumulated
235 copper.

236 Copper concentration in all biological and water samples was determined by flame
237 atomic absorption spectrometry (flame-AAS; Varian SpectrAA-250 Plus apparatus) at the
238 Scientific and Technological Research Assistance Centre (C.A.C.T.I., University of Vigo,
239 Spain) with detection limit of 0.005 mg L^{-1}.

240

241 *2.10 Data analysis*

242 Mortality of shredders was recorded, and the concentration inducing 50% of death
243 (LC_{50}) at 96 h of exposure with the respective 95% C.l. was calculated using PriProbit 1.63
244 (Sakuma, 1998; http://bru.gmprc.ksu.edu/proj/priprobit/download.asp). Repeated-measures
245 analysis of variance (ANOVA) was used to test the effects of concentrations of nano CuO on
246 the percentage of animal survival in the acute lethality test with matched observations of
247 exposure time (Zar, 2009). Two-way ANOVAs were used to determine the effects of

sublethal concentrations of nano CuO and the type of exposure (dietary or aqueous) on leaf decomposition by microbes, leaf consumption rate by invertebrates and invertebrate growth rate (Zar, 2009). Significant differences between control and treatments were analysed by Bonferroni post-tests (Zar, 2009). To achieve normal distribution and homoscedasticity, percentage data of invertebrate survival during acute tests were arcsine square root transformed and the remaining data were ln-transformed (Zar, 2009). Analyses were performed with Statistica 6.0 (Statsoft, Inc., Tulsa, OK, USA).

3. Results

3.1 Acute lethal effect of nano CuO on the invertebrate

In the acute lethality test, survival of the early-stage larvae of the invertebrate *Allogamus ligonifer* was recorded in every 24 h during 96 h of exposure to nano CuO (Fig 2). Exposure to nano CuO had a significant effect on the survival of the invertebrate larvae (repeated-measures ANOVA, $P<0.05$; Table 1). The mortality increased with increasing concentration of nano CuO and exposure time (Fig 2). The 96 h LC_{50} (95% C.l.) of nano CuO was 569 (328–1780) mg L^{-1} and the lowest observed effect concentration (LOEC) corresponded to 250 mg L^{-1} (Bonferroni test $P<0.05$).

3.2 Effects of nano CuO on leaf consumption by invertebrates and microbes

Leaf consumption rate by the early-stage larvae of *A. ligonifer* during 10 days was 0.27 mg leaf DM mg^{-1} animal DM day^{-1} in control (Fig 3) and was affected by both nanoparticle concentration and type of exposure (two-way ANOVA, $P<0.05$; Table 1). The highest inhibition was observed when animals were exposed to 75 mg L^{-1} nano CuO via stream water (0.14 mg leaf DM mg^{-1} animal DM day^{-1}, Fig 3A, Bonferroni $P<0.05$) followed by the treatment where the animals were fed on leaves pre-exposed to 75 mg L^{-1} nano CuO

(0.20 mg leaf DM mg^{-1} animal DM day^{-1}, Fig 3B, Bonferroni $P<0.05$). Leaf consumption rate was not affected by exposure to the lowest tested nano CuO concentration (25 mg L^{-1}) via water or food (Fig 3A and B, Bonferroni $P>0.05$).

Leaf decomposition rate by microbes during 10 days was 1.3 mg leaf DM $microcosm^{-1}$ day^{-1} in control, corresponding to almost 34% of the total leaf consumption rate in the presence of the invertebrate (3.84 mg leaf DM $microcosm^{-1}$ day^{-1} in control, Fig 4A, 4B). Both concentration of nano CuO and type of exposure had significant effects on microbial decomposition of leaf litter (two-way ANOVA, $P<0.05$; Table 1). Microbial decomposition rate decreased significantly after exposure to 25 and 75 mg L^{-1} nano CuO via water (Fig 4A, Bonferroni $P<0.05$) and via leaves pre-exposed to the highest nano CuO concentration (Fig 4B, Bonferroni $P<0.05$).

3.3 Effects of nano CuO on invertebrate growth

The growth rate of the invertebrate shredder was affected by the concentration of nano CuO, regardless the type of exposure, i.e. via water or food (two-way ANOVA, $P<0.05$ and $P>0.05$, respectively; Table 1). In the control, mean growth rate of the invertebrate was 56 µg animal DM mg^{-1} animal DM day^{-1}. The growth rate decreased significantly in treatments where animals were exposed for 10 days to 75 mg L^{-1} nano CuO via water (30 µg animal DM mg^{-1} animal DM day^{-1}, Fig 5A, Bonferroni $P<0.05$), followed by treatments with animals that were fed on leaves pre-exposed to 75 mg L^{-1} nano CuO (41 µg animal DM mg^{-1} animal DM day^{-1}, Fig 5B, Bonferroni $P<0.05$). Similarly to that found for invertebrate feeding rates, the exposure to the lowest tested concentration of nano CuO through water or pre-exposed leaves had no effect on animal growth rates (Fig 5A, 5B, Bonferroni $P>0.05$).

298 *3.4 Copper in water, adsorbed and accumulated in leaves and invertebrate*

299 In control, total copper and dissolved ionic copper (Cu^{2+}) in the stream water were
300 below the detection limit (<0.005 mg L^{-1}) either at the initial time (t_0) or at the end of the
301 feeding experiment (t_{10}) (Table 2). In the stream water supplemented with 25 mg L^{-1} nano
302 CuO, total Cu content varied little during the experiment (t_0, 20.98; t_{10}, 19.10 mg L^{-1}), and
303 Cu^{2+} (t_0, <0.005 mg L^{-1}) increased till 0.6 mg L^{-1} (t_{10}). In the water supplemented with 75 mg
304 L^{-1} nano CuO, total Cu decreased 7%, whereas Cu^{2+} increased 12 times. In microcosms with
305 leaves pre-exposed to nano CuO, the initial total Cu or Cu^{2+} content in water was below the
306 detection limit, and total Cu increased up to 0.476 and 1.017 mg L^{-1} for 25 and 75 mg L^{-1}
307 treatments, respectively. Cu^{2+} increased till 0.064 mg L^{-1} in water containing leaves pre-
308 exposed to 75 mg L^{-1} nano CuO, and no detectable increase was observed at the lowest tested
309 concentration.

310 After 10 days of aqueous exposure to nano CuO, the adsorbed copper was higher on
311 leaves, intermediate on the larval case and lower on the larval body (Table 2); however, in the
312 exposure via diet, the adsorbed Cu was lower on the larval case than on the larval body. The
313 accumulated copper was also higher in leaves, intermediate in larval body, and lower in the
314 larval case, regardless the route of exposure (Table 2). In all treatments, the accumulation of
315 Cu was lower than the adsorption. The content of water-soluble Cu^{2+} and water-insoluble Cu
316 adsorbed or accumulated in leaves, larval case or body increased with increasing nano CuO
317 concentration via both exposure routes, but it was higher when exposure occurred via water
318 (Table 2).

319

320 **4. Discussion**

321 Acute lethality tests are of primary importance in ecotoxicology to assess sensitivity,
322 viability and acute stress response of biota for predicting the impacts of toxicants or

323 contaminants to ecosystem functioning (Valenti et al., 2005). Although very few studies on
324 toxicity of metal oxide nanoparticles to aquatic biota are available (see Petersen and Nelson,
325 2010), acute toxicity of nano CuO to freshwater crustaceans, *Daphnia magna* and
326 *Thamnocephalus platyurus*, and to the ciliate protozoan *Tetrahymena thermophila* was shown
327 based on mobility, mortality or growth inhibition (Blinova et al., 2010). In the current study,
328 the 96 h acute lethality test on the shredder *Allogamus ligonifer* showed that this freshwater
329 invertebrate was able to survive up to 100 mg L^{-1} of nano CuO in the stream water. However,
330 survival of this species was severely affected when exposed to higher concentrations of nano
331 CuO during the acute toxicity test. Although there is no estimated or predicted data for nano
332 CuO concentration in aquatic environments, copper concentration in the chemical mechanical
333 planarization waste water of Taiwan often exceeds 100 ppm, 49% of which can be nano CuO
334 (Hsiao et al., 2001; Huang et al., 2006). Therefore, the obtained high lethal concentrations of
335 nano CuO cannot be ignored.

336 Feeding behaviour of invertebrates is one of the most accepted and sensitive
337 monitoring tools in ecotoxicology for assessing sublethal effects of metals (Pestana et al.,
338 2007) and nano metals (Buffet et al., 2011; Galloway et al., 2010). In control, the feeding rate
339 of *A. ligonifer* (0.27 mg leaf DM mg^{-1} animal DM day^{-1}) was within the typical range
340 reported for stream invertebrate shredders (0.04 to 0.5 mg leaf DM mg^{-1} animal DM day^{-1};
341 Arsuffi and Suberkropp, 1989). Using two sublethal concentrations of nano CuO (25 and 75
342 mg L^{-1}), we found that leaf consumption and growth rates of the shredder were affected by
343 the highest concentration of nano CuO through both aqueous and dietary exposure routes.
344 The outcome of our study clearly shows that the nano metal toxicity to aquatic organisms can
345 also occur via diet and not only via waterborne exposure, as often assumed for ionic metals
346 (see Brinkman and Johnston, 2008). Results also indicate that examining sublethal effects of
347 nano metals can be more rational and useful to assess toxicity than merely rely on lethal

effects. Maximum decrease in leaf consumption rate (47%) and growth rate (46%) was obtained when the animals were exposed to nano CuO via water. This agrees with the recent report on decreased feeding rates of the marine invertebrate *Scrobicularia plana* exposed to nano CuO via water (Buffet et al., 2011). In our study, the decrease in leaf consumption and invertebrate growth appeared to be lower after exposure via food than via water. However, we should point out that the pre-exposure period of food to nano CuO was not as long as the exposure of invertebrates to contaminated water. Our results encourage the use of feeding behaviour of invertebrate shredders as an endpoint for assessing toxicity of metal nanoparticles in aquatic environments.

In this study, the decrease in invertebrate feeding and growth by nano CuO exposure may be related to the food avoidance behaviour of shredders (Wilding and Maltby, 2006). Alder leaves have a high nutrient content, and leaves that are well colonized by microbes are more palatable for invertebrate shredders, including Trichoptera (Arsuffi and Suberkropp, 1989; Chung and Suberkropp, 2009; Graça, 2001). We previously reported that ionic copper (Duarte et al., 2008) and nano CuO (Pradhan et al., 2011) have negative effects on microbes colonizing leaf litter. In our study, alder leaves were pre-colonized by microbes, so leaf quality and palatability for shredders might also be affected by the impacts of nano CuO on microbial communities. Indeed, we found a severe reduction in microbial decomposition during invertebrate feeding under nano CuO exposure, particularly when exposure occurred via water. Thus, the stress induced by nanoparticles may have affected the invertebrate shredder directly or indirectly due to the effects on microbes.

Under aqueous exposure, great amounts of copper was adsorbed and accumulated in the leaves (at 75 mg L^{-1}: 15.832 and 12.889 mg g^{-1}, respectively). This was accompanied by high levels of Cu adsorption and accumulation in the larval case and body. The accumulation of Cu in the shredder body increased with the increase in CuO nanoparticle concentration in

water or food, suggesting the intake of CuO nanoparticles. The ionic counterpart of copper, leached from the CuO nanoparticles, may play an important role in enhancing the toxicity or ecotoxicity (Aruoja et al., 2009; Kahru et al., 2008; Kasemets et al., 2009). Blinova et al. (2010) using a Cu-sensor bacteria reported about 12% dissolution of Cu^{2+} from nano CuO in freshwaters. Before the feeding experiment, Cu^{2+} in water attained 0.156 mg L^{-1} in microcosms supplemented with the highest concentration of nano CuO via water. During the feeding experiment, the Cu^{2+} content increased, particularly when exposure occurred via water. Consistently, in our study, the highest levels of Cu^{2+} associated with the larval body were found after exposure to the highest sublethal concentration of nanoparticles via water followed by via food. Taking into account that toxicity of nano metals can depend on the leached ionic metal (Heinlaan et al., 2008; Mortimer et al., 2010), Cu^{2+} might have contributed to the inhibition of invertebrate feeding and growth after aqueous or dietary exposure to 75 mg L^{-1} of nano CuO. This may be a consequence of Cu^{2+} leached from nano CuO, as nanoparticles were the only source of Cu^{2+} in both exposure routes.

In our study, the leached ionic copper may have greatly contributed to the toxicity of nano CuO at lethal or sublethal concentrations. This is supported by previous studies on nano CuO toxicity to aquatic organisms including crustaceans (Blinova et al., 2010; Heinlaan et al., 2008). But further investigation is needed pertaining to the mechanisms of toxicity and other possible factors that might have involved with toxicity. Some studies reported that leached metal ions are insufficient in explaining the toxicity of nanoparticles. Griffitt et al. (2008) showed very low dissolution of nano copper that could account only for 10-15% of the toxicity to *Daphnia pulex* and zebrafish. Lower dissolution of nano CuO was reported by Buffet et al. (2011). However, the toxicity can be further argued by intracellular dissolution of nanoparticles. The oral toxicity of copper nanoparticles was attributed to the high reactivity of nano Cu that could lead to metabolic alkalosis or intracellular dissolution leading

to excessive accumulation of copper ions (Meng et al. 2007). Perhaps this is the explanatory bridge between the observed negative effects on larval feeding and growth and the high amounts of accumulated copper inside the larval body after exposure to the highest concentration of nano CuO via water or diet.

Overall, we found that copper oxide nanoparticles can have toxic effects on the invertebrate shredder *A. ligonifer*. Nanoparticle exposure led to lethal effects to this shredder only at very high concentrations. However, at sublethal levels, nano CuO was potent to decrease the feeding and growth rates of the shredder through both aqueous and dietary exposure. Results also suggested that leached ionic copper play a role in the toxicity of nano CuO, but further investigation is needed to comprehend the actual mode of action of nano-metal oxides.

Acknowledgements

FEDER-POFC-COMPETE and the Portuguese Foundation for Science and Technology supported this study (PEst-C/BIA/UI4050/2011, NANOECOTOX-PTDC/AAC-AMB/121650/2010) and A. Pradhan (SFRH/BD/45614/2008). The authors are thankful to M.J. Feio (IMAR, University of Coimbra, Portugal) for confirming invertebrate species identity.

References

Aitken, R.J., Chaudhry, M.Q., Boxall, A.B.A., Hull, M., 2006. Manufacture and use of nanomaterials: current status in the UK and global trends. Occup. Med. 56, 300–306.

Arsuffi, T.L., Suberkropp, K., 1989. Selective feeding by shredders on leaf-colonizing stream fungi: comparison of macroinvertebrate taxa. Oecologia 79, 30–37.

Aruoja, V., Dubourguier, H.C., Kasemets, K., Kahru, A., 2009. Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*. Sci. Total. Environ. 407, 1461–1468.

Becheri, A., Dürr, M., Nostro, P.L., Baglioni, P., 2008. Synthesis and characterization of zinc oxide nanoparticles: application to textiles as UV-absorbers. J. Nanopart. Res. 10, 679–689.

Blaise, C., Gagné, F., Férard, J., Eullaffroy, P., 2008. Ecotoxicity of selected nano-materials to aquatic organisms. Environ. Toxicol. 23, 591–598.

Bonada, N., Zamora-Muñoz, C., El Alami, M., Múrria, C., Prat, N., 2008. New records of Trichoptera in reference Mediterranean-climate rivers of the Iberian Peninsula and North of Africa: Taxonomical, faunistical and ecological aspects. Graellsia 64, 189–208.

Blinova, I., Ivask, A., Heinlaan, M., Mortimer, M., Kahru, A., 2010. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. Environ. Pollut. 158, 41–47.

Brinkman, S.F., Johnston, W.D., 2008. Acute toxicity of aqueous copper, cadmium, and zinc to the mayfly *Rhithrogena hageni*. Arch. Environ. Contam. Toxicol. 54, 466–472.

Buffet, P.E., Tankoua, O.F., Jin-Fen Pan, J.F., Berhanu, D., Herrenknecht, C., Poirier, L., Amiard-Triquet, C., Amiard, J.C., Bérard, J.B., Risso, C., Guibbolini, M., Roméo, M., Reip, P., Valsami-Jones, E., Mouneyrac, C., 2011. Behavioural and biochemical responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles. Chemosphere 84, 166 – 174.

Carnes, L.C., Klabunde, K.J., 2003. The catalytic methanol synthesis over nanoparticle metal oxide catalysts. J. Mol. Catal. A: Chem. 194, 227–236.

Cattaneo, A.G., Gornati, R., Chiriva-Internati, M., Bernardini, G., 2009. Ecotoxicology of nanomaterials: the role of invertebrate testing. Invertebrate Surviv. J. 6, 78–97.

Christian, P., Von der Kammer, F., Baalousha, M., Hofmann, T., 2008. Nanoparticles: structure, properties, preparation and behaviour in environmental media. Ecotoxicology 17, 326–343.

Chung, N., Suberkropp, E., 2009. Effects of aquatic fungi on feeding preferences and bioenergetics of *Pycnopsyche gentilis* (Trichoptera: Limnephilidae). Hydrobiologia 630, 257–269.

Colvin, V.L., 2003. The potential environmental impact of engineered nanomaterials. Nat. Biotechnol. 21, 1166–1170.

De Schamphelaere, K.A.C., Forrez, I., Dierckens, K., Sorgeloos, P., Janssen, C.R., 2007. Chronic toxicity of dietary copper to *Daphnia magna*. Aquat. Toxicol. 81, 409–418.

De Schamphelaere, K.A.C., Vasconcelos, F.M., Allen, H.E., Janssen, C.R., 2004. The effect of dissolved organic matter source on acute copper toxicity to *Daphnia magna*. Environ. Toxicol. Chem. 23, 1248–1255.

Duarte, S., Pascoal, C., Alves, A., Correia, A., Cássio, F., 2008. Copper and zinc mixtures induce shifts in microbial communities and reduce leaf litter decomposition in streams. Freshwat. Biol. 53, 91–101.

Duarte, S., Pascoal, C., Garabétian, F., Cássio, F., Charcosset, J.Y., 2009. Microbial decomposer communities are mainly structured by trophic status in circumneutral and alkaline streams. Appl. Environ. Microbiol. 75, 6211–6221.

Dutta, A., Das, D., Grilli, M.L., Di Bartolomeo, E., Traversa, E., Chakravorty, D., 2003. Preparation of sol–gel nano-composites containing copper oxide and their gas sensing properties. J. Sol–Gel Sci. Technol. 26, 1085–1089.

Ferreira, V., Gonçalves, A.L., Godbold, D.L., Canhoto, C., 2010. Effect of increased atmospheric CO_2 on the performance of an aquatic detritivore through changes in water temperature and litter quality. Glob. Change Biol. 16, 3284–3296.

Gajjar, P., Pettee, B., Britt, D.W., Huang, W., Johnson, W.P., Anderson, A.J., 2009. Antimicrobial activities of commercial nanoparticles against an environmental soil microbe, *Pseudomonas putida* KT2440. J. Biol. Eng. 3, pp 13, doi:10.1186/1754-1611-3-9.

Galloway, T., Lewis, C., Dolciotti, I., Johnston, B.D., Moger, J., Regoli, F., 2010. Sublethal toxicity of nano-titanium dioxide and carbon nanotubes in a sediment dwelling marine polychaete. Environ. Pollut. 158, 1748–1755.

Gerhardt, A., de Bisthoven, L.J., Soares, A.M.V.M., 2004. Macroinvertebrate response to acid mine drainage: community metrics and on-line behavioural toxicity bioassay. Environ. Pollut. 130, 263–274.

Graça, M.A.S., 2001. The role of invertebrates on leaf litter decomposition in streams – A review. Internat. Rev. Hydrobiol. 86, 383–393.

Graça, M.A.S., Canhoto, C., 2006. Leaf litter processing in low order streams. Limnetica. 25, 1–10.

Griffitt, R.J., Luo, J., Bonzongo, J.C., Barber, D.S., 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environ. Toxicol. Chem. 27, 1972–1978.

Hatakeyama, S., 1989. Effect of copper and zinc on the growth and emergence of *Epeorus latifolium* (Ephemeroptera) in an indoor model stream. Hydrobiologia 174, 17–27.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.C., Kahru, A., 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Chemosphere 71, 1308–1316.

Heinlaan, M., Kahru, A., Kasemets, K., Arbeille, B., Prensier, G., 2011. Changes in the *Daphnia magna* midgut upon ingestion of copper oxide nanoparticles: A transmission electron microscopy study. Water Res. 45, 179–190.

Hochmannova, L., Vytrasova, J., 2010. Photocatalytic and antimicrobial effects of interior paints. Prog. Org. Coat. 67, 1–5.

Hsiao, M.C., Wang, H.P., Yang, Y.W., 2001. EXAFS and XANES studies of copper in a solidified fly ash. Environ. Sci. Technol. 35, 2532–2535.

Huang, H.L., Wang, H.P., Wei, G.T., Sun, I.W., Huang, J.F., Yang, Y.W., 2006. Extraction of nanosize copper pollutants with an ionic liquid. Environ. Sci. Technol. 40, 4761–4764.

Jin, S., Ye, K., 2007. Nanoparticle-mediated drug delivery and gene therapy. Biotechnol. Prog. 23, 32–41.

Kaegi, R., Ulrich, A., Sinnet, B., Vonbank, R., Wichser, A., Zuleeg, S., Simmler, H., Brunner, S., Vonmont, H., Burkhardt, M., Boller, M., 2008. Synthetic TiO_2 nanoparticle emission from exterior facades into the aquatic environment. Environ. Pollut. 156, 233–239.

Kahru, A., Dubourguier, H.C., Blinova, I., Ivask, A., Kasemets, K., 2008. biotests and biosensors for ecotoxicology of metal oxide nanoparticles: A minireview**.** Sensors 8, 5153–5170.

Karlsson, H.L., Gustafsson, J., Cronholm, P., Möller, L., 2009. Size-dependent toxicity of metal oxide particles–A comparison between nano- and micrometer size. Toxicol. Lett. 188, 112–118.

Kasemets, K., Ivask, A., Dubourguier, H.C., Kahru, A., 2009. Toxicity of nanoparticles of ZnO, CuO and TiO_2 to yeast *Saccharomyces cerevisiae*. Toxicol. in Vitro 23, 1116–1122.

Kathirvelu, S., D'Souza, L., Dhurai, B., 2009. UV protection finishing of textiles using ZnO nanoparticles. Indian J. Fibre Text. 34, 267–273.

Lee, S.W., Kim, S.M., Choi, J., 2009. Genotoxicity and ecotoxicity assays using the freshwater crustacean *Daphnia magna* and the larva of the aquatic midge *Chironomus*

riparius to screen the ecological risks of nanoparticle exposure. Environ. Toxicol. Pharmacol. 28, 86–91.

Lovern, S.B., Strickler, J.R., Klaper, R., 2007. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C_{60}, and $C_{60}HxC_{70}Hx$). Environ. Sci. Technol. 41, 4465–4470.

MacCormack, T.J., Goss, G.G., 2008. Identifying and predicting biological risks associated with manufactured nanoparticles in aquatic ecosystems. J. Ind. Ecol. 12, 286–296.

Meng, H., Chen, Z., Xing, G., Yuan, H., Chen, C., Zhao, F., Zhang, C., Zhao, Y., 2007. Ultrahigh reactivity provokes nanotoxicity: Explanation of oral toxicity of nanocopper particles. Toxicol. Lett. 175, 102–110.

Miller, R.J., Lenihan, H.S., Muller, E.B., Tseng, N., Hanna, S.K., Keller, A.A., 2010. Impacts of metal oxide nanoparticles on marine phytoplankton. Environ. Sci. Technol. 44, 7329–7334.

Moore, M.N., 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environ. Int. 32, 967–976.

Mortimer, M., Kasemets, K., Kahru, A., 2010. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. Toxicology 269, 182–189.

Navarro, E., Baun, A., Behra, R., Hartmann, N.B., Filser, J., Miao, A.J., Quigg, A., Santschi, P.H., Sigg, L., 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 17, 372–386.

Nel, A., Xia, T., Mädler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science 311, 622–627.

OECD, 2010. Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's sponsorship programme, ENV/JM/MONO(2009)20REV. OECD Environment, Health and Safety Publications, Series on the Safety of Manufactured Nanomaterials No. 25, Organisation for Economic Co-operation and Development, Paris.

Pascoal, C., Cássio, F., Gomes, P., 2001. Leaf breakdown rates: a measure of water quality? Int. Rev. Hydrobiol. 86, 407–416.

Pestana, J.L.T., Ré, A., Nogueira, A.J.A., Soares, A.M.V.M., 2007. Effects of cadmium and zinc on the feeding behaviour of two freshwater crustaceans: *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda). Chemosphere 68, 1556–1562.

555 Petersen, E.J., Nelson, B.C., 2010. Mechanisms and measurements of nanomaterial-
556 induced oxidative damage to DNA. Anal. Bioanal. Chem. 398, 613–650.
557 Pradhan, A., Seena, S., Pascoal, C., Cássio, F., 2011. Can metal nanoparticles be a
558 threat to microbial decomposers of plant litter in streams? Microb. Ecol. 62, 58–68.
559 Reijnders, L., 2006. Cleaner nanotechnology and hazard reduction of manufactured
560 nanoparticles. J. Clean. Prod. 14, 124–133.
561 Ren, G., Hu, D., Cheng, E.W.C., Vargas-Reus, M.A., Reip, P., Allaker, R.P., 2009.
562 Characterisation of copper oxide nanoparticles for antimicrobial applications. Int. J.
563 Antimicrob. Agents 33, 587–590.
564 Saison, C., Perreault, F., Daigle, J.C., Fortin, C., Claverie, J., Morin, M., Popovic, R.,
565 2010. Effect of core-shell copper oxide nanoparticles on cell culture morphology and
566 photosynthesis (photosystem II energy distribution) in the green alga, *Chlamydomonas*
567 *reinhardtii*. Aquat. Toxicol. 96, 109–114.
568 Sakuma, M., 1998. Probit analysis of preference data. Appl. Entomol. Zool. 33, 339–
569 347.
570 Sharma, V.K., 2009. Aggregation and toxicity of titanium dioxide nanoparticles in
571 aquatic environment – a review. J. Environ. Sci. Health A. 44, 1485–1495.
572 Suberkropp, K., Arsuffi, T.L., Anderson, J.P., 1983. Comparison of degradative
573 ability, enzymatic activity, and palatability of aquatic hyphomycetes grown on leaf litter.
574 Appl. Environ. Microbiol. 46, 237–244.
575 Valenti, W., Chaffin, J.L., Cherry, D.S., Schreiber, M.E., Valett, H.M., Charles, M.,
576 2005. Bioassessment of an appalachian headwater stream influenced by an abandoned arsenic
577 mine. Arch. Environ. Contam. Toxicol. 49, 488–496.
578 Van Hoecke, K., Quik, J.T., Mankiewicz-Boczek, J., De Schamphelaere, K.A.,
579 Elsaesser, A., Van der Meeren, P., Barnes, C., McKerr, G., Howard, C.V., Van de Meent, D.,
580 Rydzyński, K., Dawson, K.A., Salvati, A., Lesniak, A., Lynch, I., Silversmit, G., De Samber,
581 B., Vincze, L., Janssen, C.R., 2009. Fate and effects of CeO_2 nanoparticles in aquatic
582 ecotoxicity tests. Environ. Sci. Technol. 43, 4537-4546.
583 Varandas, S.G., Cortes, R.M.V., 2010. Evaluating macroinvertebrate biological
584 metrics for ecological assessment of streams in northern Portugal. Environ. Monit. Assess.
585 166, 201–221.

586 Wang, Z., Lee, Y.H., Wu, B., Horst, A., Kang, Y., Tang, Y.J., Chen, D.R., 2010.
587 Anti-microbial activities of aerosolized transition metal oxide nanoparticles. Chemosphere
588 80, 525–529.
589 Wilding, J., Maltby, L., 2006. Relative toxicological importance of aqueous and
590 dietary metal exposure to a freshwater crustacean: implication for risk assessment. Environ.
591 Toxicol. Chem. 25, 1795–1801.
592 Zar, J.H., 2009. Biostatistical Analysis, fifth ed. Prentice-Hall, Upper Saddle River,
593 New Jersey.
594 Zhang, X., Zhang, D., Ni, X., Song, J., Zheng, H., 2008. Synthesis and
595 electrochemical properties of different sizes of the CuO particles. J. Nanopart. Res. 10, 839–
596 844.
597 Zhu, X., Zhu, L., Duan, Z., Qi, R., Li, Y., Lang, Y., 2008. Comparative toxicity of
598 several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early
599 developmental stage. J. Environ. Sci. Health A. 43, 278–284.
600
601
602

603

604 Table 1 ANOVAs on effects of nano CuO concentration on invertebrate survival, and effects of exposure type (dietary or aqueous)
605 and nano CuO concentration on leaf decomposition by microbes, leaf consumption by the invertebrate shredder and growth rates
606 of the shredder *Allogamus ligonifer*.

Parameter	ANOVA	Effect	d.f.	*F*	*P*-value
Invertebrate survival	Repeated-measures	Nano CuO concentration	5	11.45	<0.0001
Leaf decomposition by microbes	Two-way	Exposure type	1	7.828	<0.01
		Nano CuO concentration	2	16.43	<0.0001
		Exposure type * Nano CuO concentration	2	2.219	>0.05
Leaf consumption by invertebrate	Two-way	Exposure type	1	4.425	<0.05
		Nano CuO concentration	2	13.46	<0.0001
		Exposure type * Nano CuO concentration	2	2.973	>0.05
Invertebrate growth	Two-way	Exposure type	1	1.569	>0.05
		Nano CuO concentration	2	12.96	<0.0001
		Exposure type * Nano CuO concentration	2	1.314	>0.05

607 d.f., degree of freedom.

608

609

610

Table 2 Total and ionic copper concentrations in water, and adsorbed and accumulated in leaves and invertebrates after 10 days (t_{10}) exposure to nano CuO via food or water. Exposure conditions: via food - leaves were pre-exposed for 5 days to nano CuO and then released from nano CuO for further 10 days; via water - leaves were in stream water for 5 days and then were exposed to nano CuO for further 10 days.

Sample	Concentration	Control	Nano CuO exposure			
			Via water		Via food	
	Added nano CuO (mg L^{-1})	0	25	75	25	75
Water	Cu (mg L^{-1})	t_0 / t_{10}	t_0 / t_{10}	t_0 / t_{10}	t_0 / t_{10}	t_0 / t_{10}
	Total Cu	nd/nd	20.982/ 19.1	61.167/ 56.857	nd/0.476	nd/1.017
	Cu^{2+}	nd/nd	nd/0.6	0.156/1.87	nd/nd	nd/0.064
	[a] % of Cu^{2+}	-	nd/3.141	0.255/3.289	-	nd/6.293
Leaves	Cu after exposure (mg g^{-1})					
	Adsorbed Cu	0.007	11.676	15.832	2.359	3.316
	Accumulated Cu	0.003	4.086	12.889	0.485	1.229
	Cu^{2+}	nd	0.076	0.113	0.011	0.013
	[b] % of Cu^{2+}	-	0.48	0.392	0.385	0.285
Larval case	Cu after exposure (mg g^{-1})					
	Adsorbed Cu	0.021	1.394	3.112	0.064	0.162
	Accumulated Cu	0.013	0.162	0.619	0.016	0.021
	Cu^{2+}	0.003	0.084	0.158	0.005	0.013
	[b] % of Cu^{2+}	8.108	5.122	4.063	5.882	6.633
Larval body	Cu after exposure (mg g^{-1})					
	Adsorbed Cu	0.011	0.893	2.962	0.241	0.529
	Accumulated Cu	0.052	0.3	0.942	0.14	0.496
	Cu^{2+}	nd	0.065	0.331	0.035	0.116
	[b] % of Cu^{2+}	-	5.167	7.816	8.413	10.167

nd: below detection limit.

[a] With respect to total copper (suspended nano and dissolved ionic forms).

[b] With respect to total copper (adsorbed, accumulated and ionic).

620

621 **Figure legends:**

622 **Figure 1** Size distribution of nano CuO in stock suspension by dynamic light scattering.

623

624 **Figure 2** Acute lethal toxicity of nano CuO to early-stage larvae of the invertebrate
625 *Allogamus ligonifer* with respect to time.

626

627 **Figure 3** Leaf consumption rates by the early-stage larvae of *Allogamus ligonifer* for 10 days at 14
628 ºC. The animals were exposed to nano CuO through contaminated stream water (A), or through
629 contaminated leaves (B). Mean ± SEM, n=15. *, treatments that differ significantly from control
630 (Bonferroni tests, $P<0.05$).

631

632 **Figure 4** Total leaf consumption by the shredder *Allogamus ligonifer* (dark grey bars) and
633 microbial decomposition of leaf litter (light grey bars) during 10 days in microcosms at 14
634 ºC. The animals and microbes were exposed to nano CuO through contaminated stream water
635 (A), or through contaminated leaves (B). Mean ± SEM, n=15. *, treatments that differ
636 significantly from control (Bonferroni tests, $P<0.05$).

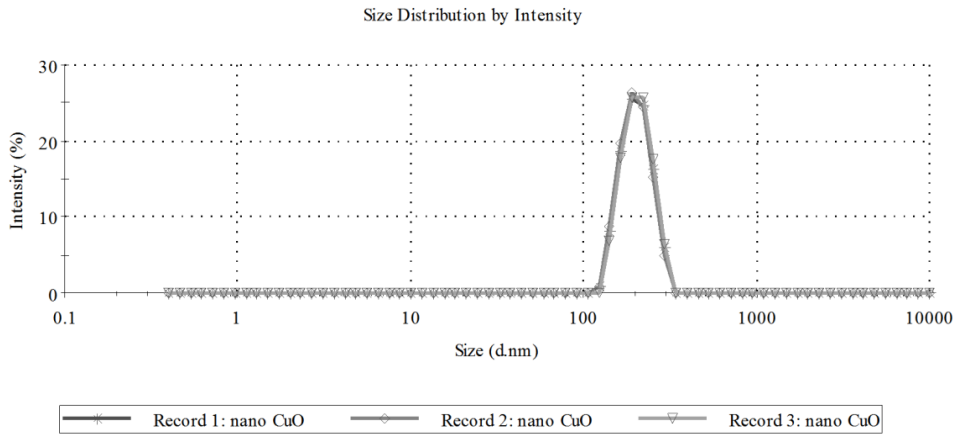
637

638 **Figure 5** Growth rates of the early-stage larvae of *Allogamus ligonifer* feeding on microbially-
639 colonized leaves for 10 days at 14 ºC. The animals were exposed to nano CuO through
640 contaminated stream water (A), or through contaminated leaves (B). Mean ± SEM, n=15. *,
641 treatments that differ significantly from control (Bonferroni tests, $P<0.05$).

642

643

644



645

646 **Fig 1**

647

648

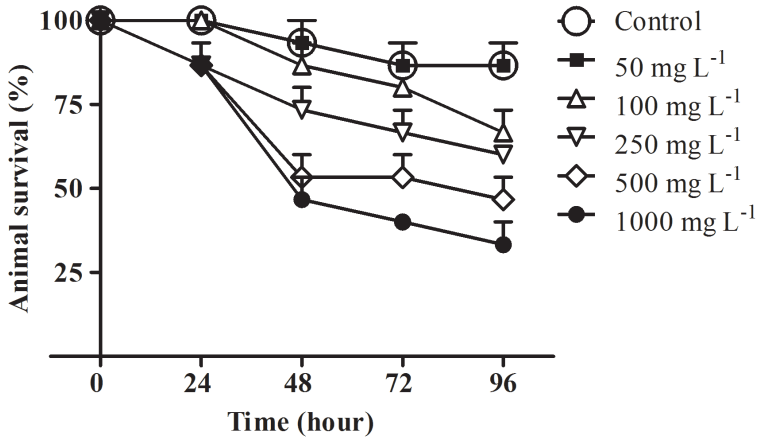


Fig 2

649

650

651

652

653

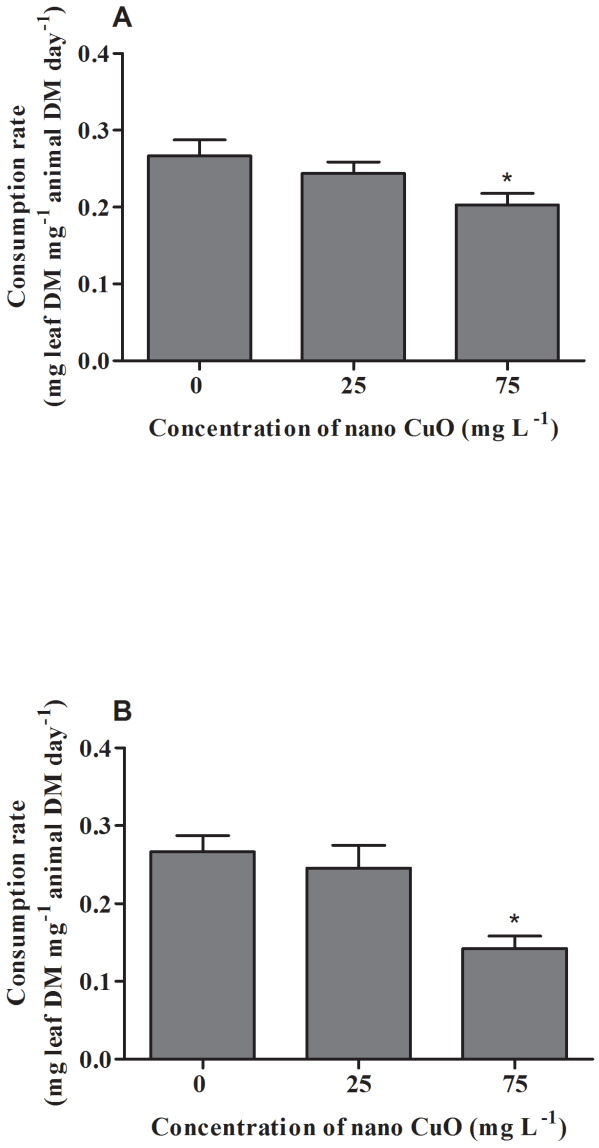


Fig 3

654

655

656

657

658

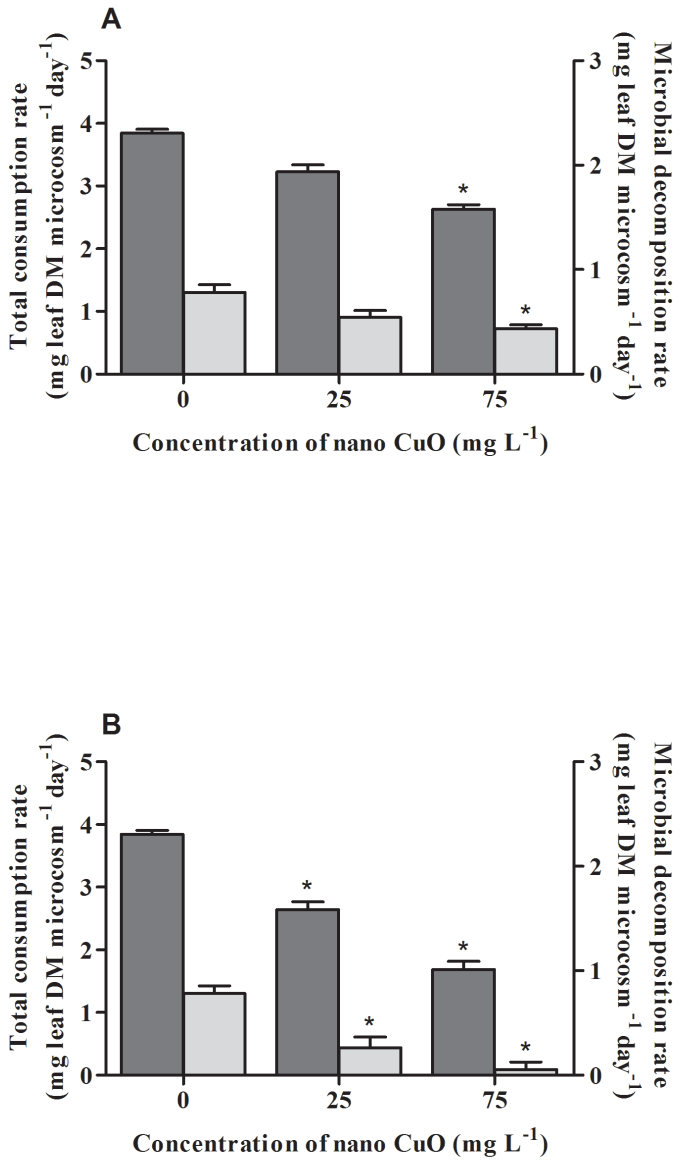


Fig 4

659

660

661

662

663

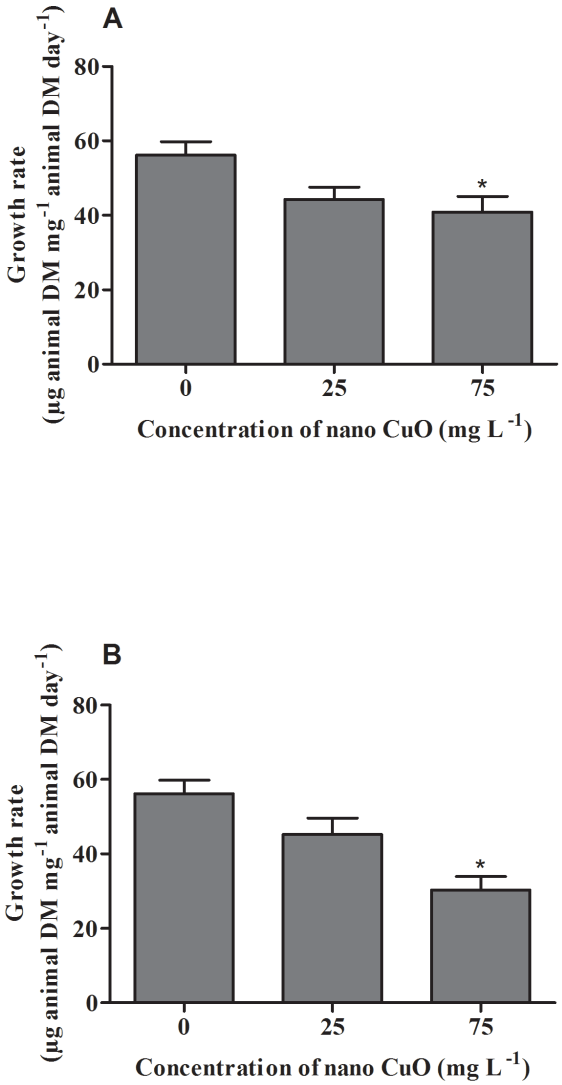


Fig 5

664