Copper oxide nanoparticles can induce toxicity to the freshwater shredder *Allogamus ligonifer*. Chemosphere 89: 1142-1150.
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

Nanoeotoxicology 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, Ca²⁺ 1.3 ± 0.3 mg L⁻¹, K⁺ 0.6 ± 0.1 mg L⁻¹, HCO₃⁻ 8.0 ± 0.8 mg L⁻¹, Cl⁻ 4.2 ± 0.4 mg L⁻¹, and SO₄²⁻ 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

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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
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Pradhan A, Seena S, Pascoal C, Cássio F (2012) 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

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

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

### 2.10 Data analysis

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

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(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 \(\mu\)g 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 \(\mu\)g 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 \(\mu\)g 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 \(P>0.05\)).
3.4 Copper in water, adsorbed and accumulated in leaves and invertebrate

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

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

4. Discussion

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

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

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

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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.

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Copper oxide nanoparticles can induce toxicity to the freshwater shredder *Allogamus ligonifer*.

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ANOVA Effect</th>
<th>d.f.</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invertebrate survival</td>
<td>Repeated-measures Two-way</td>
<td>5</td>
<td>11.45</td>
<td>&lt;0.0001</td>
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<tr>
<td>Leaf decomposition by microbes</td>
<td>Two-way Exposure type</td>
<td>1</td>
<td>4.425</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Leaf consumption by invertebrate</td>
<td>Two-way Exposure type</td>
<td>1</td>
<td>2.219</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Leaf consumption by invertebrate</td>
<td>Two-way Nano CuO concentration</td>
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<td>13.46</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Leaf consumption by invertebrate</td>
<td>Two-way Exposure type * Nano CuO concentration</td>
<td>2</td>
<td>2.973</td>
<td>&gt;0.05</td>
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<tr>
<td>Leaf decomposition by microbes</td>
<td>Two-way Exposure type</td>
<td>1</td>
<td>2.973</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Leaf consumption by invertebrate</td>
<td>Two-way Exposure type</td>
<td>1</td>
<td>1.569</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Leaf consumption by invertebrate</td>
<td>Two-way Nano CuO concentration</td>
<td>2</td>
<td>12.96</td>
<td>&lt;0.0001</td>
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<tr>
<td>Leaf consumption by invertebrate</td>
<td>Two-way Exposure type * Nano CuO concentration</td>
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<td>1.314</td>
<td>&gt;0.05</td>
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<td>Leaf consumption by invertebrate</td>
<td>Two-way Exposure type</td>
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<td>4.425</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Leaf consumption by invertebrate</td>
<td>Two-way Nano CuO concentration</td>
<td>2</td>
<td>11.96</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Leaf consumption by invertebrate</td>
<td>Two-way Exposure type * Nano CuO concentration</td>
<td>2</td>
<td>2.219</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Table 1 ANOVAs on effects of CuO concentration on invertebrate survival and effects of exposure type (dietary or aqueous) on leaf decomposition by microbes, leaf consumption by invertebrates, and growth rates of the shredder *Allogamus ligonifer*. Copper oxide nanoparticles can induce toxicity to the freshwater shredder *Allogamus ligonifer*. *Pradhan A, Seena S, Pascoal C, Cássio F.* (2012) *Chemosphere* 89, 1142-1150.
Copper oxide nanoparticles can induce toxicity to the freshwater shredder *Allogamus ligonifer*. *Chemosphere* 89: 1142-1150.

### Table 2

<table>
<thead>
<tr>
<th>Exposure Conditions</th>
<th>Added nano CuO (mg L⁻¹)</th>
<th>Total Cu (mg L⁻¹)</th>
<th>Cu²⁺ (mg L⁻¹)</th>
<th>Adsorbed Cu</th>
<th>Accumulated Cu</th>
<th>% of Cu²⁺</th>
<th>% of Cu</th>
<th>Cu after exposure (mg g⁻¹)</th>
<th>Adsorbed Cu</th>
<th>Accumulated Cu</th>
<th>% of Cu²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>via water</td>
<td>25</td>
<td>11.67</td>
<td>nd</td>
<td>40.64</td>
<td>11.22</td>
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<td>0.476</td>
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<td>via food</td>
<td>75</td>
<td>20.982</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.48</td>
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<td>0.003</td>
<td>4.086</td>
<td>nd</td>
<td>0.48</td>
</tr>
</tbody>
</table>

nd: below detection limit.

(a) With respect to total copper (adsorbed, accumulated and ionic).

(b) With respect to total copper (adsorbed and dissolved ionic forms).

<table>
<thead>
<tr>
<th>Exposure Conditions</th>
<th>Added nano CuO (mg L⁻¹)</th>
<th>Total Cu (mg L⁻¹)</th>
<th>Cu²⁺ (mg L⁻¹)</th>
<th>Adsorbed Cu</th>
<th>Accumulated Cu</th>
<th>% of Cu²⁺</th>
<th>% of Cu</th>
<th>Cu after exposure (mg g⁻¹)</th>
<th>Adsorbed Cu</th>
<th>Accumulated Cu</th>
<th>% of Cu²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>via water</td>
<td>25</td>
<td>5.167</td>
<td>nd</td>
<td>5.122</td>
<td>1.017</td>
<td>0.064</td>
<td>1.017</td>
<td>0.002</td>
<td>4.13</td>
<td>nd</td>
<td>0.116</td>
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<tr>
<td>via food</td>
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<td>5.02</td>
<td>nd</td>
<td>5.017</td>
<td>1.017</td>
<td>0.064</td>
<td>1.017</td>
<td>0.002</td>
<td>4.13</td>
<td>nd</td>
<td>0.116</td>
</tr>
</tbody>
</table>

nd: below detection limit.

(a) With respect to total copper (adsorbed, accumulated and ionic).

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

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**Figure legends:**

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

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

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

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

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

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Fig 1

Fig 2

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Fig 3

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![Graph A](image1)

![Graph B](image2)

Fig 5