

## Acute and chronic effects from pulse exposure of D. magna to silver and copper oxide nanoparticles

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#### ABSTRACT

Aquatic toxicity testing of nanoparticles (NPs) is challenged by their dynamic behavior in test 19 suspensions. The resulting difficulties in controlling and characterizing exposure concentrations are 20 detrimental to the generation of concentration-response data needed for hazard identification of 21 NPs. This study explores the applicability of short-term (1, 2 and 3 h) pulse exposures as means to 22 keep the exposure stable and at the same time disclose acute and chronic effects of AgNPs and 23 CuONPs in *D. magna*. Dissolution, agglomeration and sedimentation were found to have less 24 influence on exposure concentrations during 1-3 h pulses than for 24-48 h continuous exposures. 25 For AgNPs, preparation of test suspensions in medium 24 h before toxicity testing (aging) increased 26 stability during the short-term pulses. In pulse tests, organisms were exposed to the test materials 27 AgNPs and CuONPs for 1, 2 and 3 h, and afterwards transferred to clean medium and observed for 28 48 h (post-exposure period) for acute effects and for 21 d for chronic effects. AgNO<sub>3</sub> and CuCl<sub>2</sub> 29 were used as reference materials for dissolved silver and copper, respectively. For all test materials, 30 a 3 h pulse caused comparable immobility in D. magna (observed after 48 h post-exposure) as 24 h 31 continuous exposure, as evidenced by overlapping 95% confidence intervals of EC<sub>50</sub>-values. In the 32 21 d post-exposure period, no trends in mortality or body length were identified. AgNPs and 33 AgNO<sub>3</sub> pulses had no effect on the number of moltings, days to first live offspring or cumulated 34 number of offspring, but the number of offspring increased for AgNPs (3 h pulse only). In contrast, 35 CuONPs and CuCl<sub>2</sub> pulses decreased the number of moltings and offspring, and for CuONPs the 36 37 time to first live offspring was prolonged. After CuONP exposures, the offspring production decreased more with increasing concentrations than for CuCl<sub>2</sub> exposures when taking the measured 38 dissolved copper into account. This indicates a nanoparticle-specific effect for CuONPs, possible 39 40 related CuONPs accumulated in the gut of *D. magna* during the pulse exposure. Pulse exposure is an environmentally relevant exposure scenario for NPs, which for AgNPs and CuONPs enables 41 more stable exposures and cause acute immobility of D. magna comparable to continuous 24 h 42 exposures. Pulse exposure is likely relevant and applicable for other toxic and dissolving metal NPs, 43 but this requires further research. 44

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### 46 Keywords

47 Nanoecotoxicology \* Pulse exposure \* Hazard identification \* Exposure control \* Endpoints

#### 48 1. Introduction

49

Within recent decades, engineered nanoparticles (ENPs) have been increasingly produced and used 50 in products already available on the market. Silver nanoparticles (AgNPs) are the most commonly 51 applied of ENPs, utilized mainly for antimicrobial purposes in various product categories (Hansen 52 et al., 2016; SCENIHR, 2014). However, the use of other metal NPs, such as CuO, for biocidal 53 purposes is also increasing (Brinch et al., 2016). Efforts to determine potential environmental 54 hazards of ENPs within various regulatory schemes rely on the use of standard aquatic toxicity 55 tests, which have been developed and utilized for hazard identification of soluble chemicals. 56 However, standardized ecotoxicity testing of ENPs is challenged by poor reproducibility, altered 57 concentration-response patterns, and occurrence of physical effects resulting from mechanical 58 interference of the ENPs with the test system, endpoint or organism (Handy et al., 2012a; Hartmann 59 et al., 2013, 2010; Petersen et al., 2014; Sørensen et al., 2015). A critical issue in this regard is the 60 61 assumption of complete solubility and stability of the test chemical during incubation. This assumption is violated when ENPs are tested, as they by definition suspend in aqueous media rather 62 than dissolve, and furthermore exhibit highly dynamic behavior, i.e. change their physical 63 appearance both spatially and temporally (Handy et al., 2012b; Petersen et al., 2014; Sørensen and 64 Baun, 2015; Sørensen et al., 2015). 65

66

The freshwater crustacean *Daphnia magna* is commonly used for standardized toxicity testing of 67 24-48 h acute immobility and 21-days chronic effects regarding reproduction, growth and mortality 68 (OECD, 2012, 2004). Daphnids have high ecological relevance and a relatively short life-cycle that 69 70 enables chronic studies within a practical and reasonable testing timeframe of 21 days, in addition to being easily managed in the laboratory. Generally, far less studies in the scientific literature focus 71 72 on the chronic effects of ENPs in *D. magna* as opposed to acute effects, although the importance and relevance of investigating chronic effects of ENPs have been pinpointed (Wang et al., 2015; 73 Zhao and Wang, 2011). One reason is likely that chronic tests are more labor and cost intensive, 74

75 especially for ENPs, as prolonged exposure magnify the efforts and logistics needed to monitor and maintain stable exposure conditions (Handy et al., 2012a). The test suspensions in chronic tests with 76 D. magna are usually renewed every second to third day, to keep the exposure concentration stable, 77 ensure sufficient amounts of nutrients and remove waste products. For ENPs, small changes to the 78 stock preparation procedure may influence the prepared suspensions (Hartmann et al., 2015) and 79 compromise the repeatability and reproducibility of the renewal steps. For the ENPs that undergo 80 dissolution, such as AgNPs and CuONPs the issue of unstable exposure scenarios becomes further 81 complicated by the dissolution kinetics. Also, the necessity of feeding during a chronic test becomes 82 an influencing factor, as the feeding conditions have shown to influence the chronic effects of 83 AgNPs to D. magna (Mackevica et al., 2015). 84

85

Short-term exposures in D. magna identified as "pulse" or "intermittent" exposures have been 86 applied for toxicity testing of both organic and inorganic substances, with the effects being 87 monitored immediately after or during a post-exposure period in clean media after the pulse 88 (Andersen et al., 2006; Hoang and Klaine, 2008; Trac et al., 2015). Pulse toxicity tests have been 89 applied mainly to simulate a more environmentally realistic and relevant exposure scenario, as 90 many chemical pollutants are discharged into aquatic environments as "pulses" resulting from e.g. 91 92 surface runoff after rain events, overflow of wastewater treatment plants, agrochemicals and veterinary pharmaceuticals from agriculture (e.g. Handy, 1994; Hommen et al., 2010). Furthermore, 93 94 agrochemicals with a short half-life in the environment have also been suggested to exhibit pulselike exposures (Reinert et al., 2002). Analogously, ENPs are also expected to rapidly transform 95 from their pristine form, due to for example dissolution, agglomeration, sedimentation, and coating 96 97 alterations once discharged into the environment (Lowry et al., 2014) or even when added to simplified media in laboratory toxicity tests (Petersen et al., 2014). A study of stream mesocosms 98 has found the fate of cerium oxide ENPs to differ for press and pulse exposures and the authors 99 100 recommend environmental risk assessment of ENPs to address the implications of exposure duration (Baker et al., 2016). To date, several studies have investigated the chronic effects in 101

102 crustaceans exposed to ENPs of silver (Blinova et al., 2013; Gaiser et al., 2011; Mackevica et al., 2015; Pokhrel and Dubey, 2012; Seitz et al., 2015; Zhao and Wang, 2011), TiO<sub>2</sub> (Seitz et al., 2013), 103 ZnO (Adam et al., 2014; Zhao et al., 2012), and CuO (Adam et al., 2015; Rossetto et al., 2014; 104 Zhao et al., 2012). These studies focus on a variety of influencing factors such as feeding 105 conditions, exposure routes, exposure scenarios (semi-static vs. flow-through), mixture effects, ENP 106 characteristics (e.g. size, agglomeration, coating), environmental factors (e.g. pH, media, dissolved 107 organic matter) and effects of ENPs vs. released ions. However, to our knowledge, the influence of 108 exposure duration, in terms of a short-term NP pulse, and the following acute and chronic effect to 109 D. magna remains unexamined. 110

111

The aim of this study is to explore the applicability of a short-term pulse exposure to reveal acute 112 and chronic effects of Ag and CuONPs to D. magna. We exposed neonate D. magna to 1, 2 and 3 h 113 pulse exposures to AgNPs, CuONPs, silver nitrate (AgNO<sub>3</sub>) and copper chloride (CuCl<sub>2</sub>) and 114 studied the acute (immobility) and chronic effects (mortality, body length, molting and 115 reproduction) occurring within 48 h and 21 d post-exposure periods, respectively. The obtained 116 responses were then compared to those of continuous exposures in acute and chronic tests with the 117 same materials. Pulsed exposure presents an environmentally relevant exposure scenario and may 118 also facilitate more stable exposure conditions for ENPs during testing. As the transformation 119 processes of suspended ENPs are time-dependent, we expected less impact of such processes during 120 121 a short-term pulse, than during 24 or 48 h continuous exposure applied for standard acute tests. Therefore, the stability of the test suspensions in terms of agglomeration, dissolution and 122 sedimentation of AgNPs and CuONPs was monitored over the course of the pulse (1-3 h) and 123 continuous (48 h) exposures. 124

#### 125 2. Materials and Methods

126

#### 127 **2.1** Media, chemicals and preparation of suspensions

Citrate stabilized AgNPs were purchased from Cline Scientific AB (Gothenburg, Sweden) as an 128 aqueous suspension of 20 mg Ag/L in Milli-Q water containing < 0.005% Tannic acid and < 0.05%129 Trisodium citrate dehydrate. The reported silver content was confirmed by inductively coupled 130 plasma - optical emission spectrometry (Varian Vista-MPX CCD simultaneous ICP-OES). The 131 primary size determined by Transmission Electron Microscopy (TEM) for the delivered suspension 132 is  $29.9 \pm 4.5$  nm (data provided by the manufacturer). CuO nanopowder of average size  $\leq 50$  nm 133 (TEM) was purchased from Sigma-Aldrich. Silver nitrate (>99.0%, Sigma-Aldrich) and copper(II) 134 chloride, dihydrate (98%, Acros Organics) were included as soluble silver and copper controls. 135 Elendt M7 medium (OECD, 2012) with modifications, was used for *D. magna* cultivation and 136 toxicity testing (composition provided in SI, Table S1). 137

138

AgNP test suspensions were prepared by diluting the purchased suspension with medium, without 139 stirring/sonication. Suspensions were prepared in volumetric flasks and, for the pulse tests, stored in 140 the dark at 20 °C for 24 hours prior to toxicity testing. This "aging" step was conducted to have the 141 initial rapid dissolution of silver ions into medium occur before rather than during toxicity testing, 142 in accordance with previous work (Sørensen and Baun, 2015). For the toxicity test with continuous 143 exposure, AgNP suspensions were prepared fresh, as prescribed by the ISO protocol (ISO, 1989). 144 CuONP suspensions were prepared immediately prior to testing as the aging step markedly 145 decreased toxicity. This decreased toxicity was likely due to adhesion of CuONPs to container walls 146 at the suspension surface during the aging steps after the aging step, large colored stains was visible 147 on containers in the initial range finding tests (data not shown). CuO nanopowder were dispersed in 148 Milli-Q water and probe sonicated (Branson Digital Sonifier Model S-250D) in an ice cooled 149 beaker for 5 min at 10% amplitude. Polypropylene flasks and beakers were applied, as this lead to 150 greatest recoveries of Cu (data not shown). 151

#### **2.2 Characterization of test suspensions**

154 The total silver and copper concentrations were determined by ICP-MS (Agilent 7700, Agilent,

155 Santa Clara, CA, USA), and for some silver samples ICP-OES (Varian Vista-MPX CCD

156 simultaneous ICP-OES). Measurements were conducted for all test concentrations prior to toxicity

157 testing and for the highest test concentrations after ended pulse exposures. Samples were added

158 concentrated HNO<sub>3</sub> to a concentration of 17% (AgNPs) and 6.2% (CuONPs) and digested for 24

hours at 20 °C in the dark, then diluted with de-ionized water and kept in the dark at 4 °C until
analysis.

161

The particle size distribution, hydrodynamic diameter and zeta potential of NP suspensions were determined by Dynamic Light Scattering (DLS) using a Malvern ZetaSizer Nano ZS (Malvern Instruments, UK). Measurements were conducted on 1 mL sample in standard disposable cuvettes (or capillary cells for zeta potential determinations) at 25 °C with a scattering angle of 173 °. Suspensions were characterized immediately upon preparation, and at the beginning and end of toxicity test exposures.

168

The degree of sedimentation in CuONP suspensions over time was assessed in suspensions of 1.3 and 20 mg Cu/L. Carefully, without stirring the suspensions, a sample for ICP analysis was drawn after the pulse exposures (1, 2 and 3 hours) from the middle of the suspension. Then suspensions were stirred and another sample was drawn to yield the suspended and total Cu concentrations, respectively.

174

175 The concentration of dissolved Ag and Cu from NP suspensions in medium was determined by

ultracentrifugation (Beckman L8-60M). Briefly, suspensions of AgNP (10, 50 and 200  $\mu$ g Ag/L

nominal) and CuONP (0.2, 2 and 20 mg Cu/L nominal) were prepared in medium and kept in the

dark at 20 °C, similar to the exposure conditions of toxicity testing and aging. Duplicate samples of

10 mL were drawn from each suspension at times reflecting the pulse and continuous exposure
durations (and for AgNPs also the aging step before testing); For CuONPs at 0, 1, 2, 3, 24 and 48
hours and for AgNPs at 0, 24, 25, 26, 27, and 48 h. The samples were centrifuged for 30 min
(AgNPs) and 45 min (CuONPs) at 30000 rpm (~ 68000 × g) to ensure settling of particles according
to their sizes (based on calculations derived from Stoke's law and material density). The
supernatant (5 mL) was collected, digested with HNO<sub>3</sub> and the Ag and Cu concentration determined
by ICP-MS.

Speciation of Ag and Cu from AgNO<sub>3</sub> and CuCl<sub>2</sub> in modified M7 was estimated by the chemical
speciation modeling program Visual MINTEQ 3.0 (Gustafsson, 2010), assuming equilibrium with
atmospheric CO<sub>2</sub>.

189

#### 190 **2.3 Acute toxicity tests**

Acute immobilization tests were conducted with 48 hours continuous exposure in accordance with 191 OECD Guideline (OECD, 2004). Immobility was recorded after 24 and/or 48 hours. Furthermore, 192 the study included a series of tests with single pulse exposures of 1, 2 and 3 hours, followed by a 48 193 h post-exposure observation period in clean medium. All tests included a control and five exposure 194 concentrations with four replicates, each comprised of five neonates (< 24 h old) in 25 mL test 195 suspension contained in 100 ml beakers. All exposures and post-exposure incubations were carried 196 out at  $20 \pm 2$  °C in the dark. For the pulse tests, daphnids were rinsed in medium at the end of the 197 198 exposure time and then transferred to beakers with clean medium for the 48 h post-exposure period. Immobility was recorded immediately upon transfer (0 hours) and after 24 and 48 hours. An 199 overview of applied concentration ranges for the toxicity tests are given in Table 1. The pH of all 200 test concentrations remained within 7.7-8.9 during exposure and post-exposure. The validity criteria 201 were met for all acute tests, with no control mortalities and an oxygen content > 9 mg/L. 202 203

204 **2.4 Chronic toxicity tests** 

The chronic toxicity following a single pulse exposure to the test materials was investigated over a 21 days post-exposure observation period. The tests were carried out in accordance with OECD 207 guideline (OECD, 2012), however all organisms were kept in clean media throughout the 208 observation period. Ten neonate daphnids (< 24 h old) was exposed in a 100 mL beaker containing 209 50 mL test suspension (or medium for the controls) in the dark at  $20 \pm 2$  °C. The exposure 210 concentration ranges (Table 1) were based on the outcome of the acute toxicity tests, to target a 211 maximum 10% lethality.

Upon pulse exposures, the ten neonates in each exposure group were rinsed shortly in medium and 212 transferred to ten separate beakers of 50 mL medium for the 21-d post-exposure period. During the 213 post-exposure, daphnids were kept in the climate controlled room, also used for the general 214 cultivation of *D. magna*, at  $20 \pm 2$  °C with a 16:8 h light:dark cycle. Daphnids were daily fed a 215 concentrated algal suspension (*P. subcapitata*) of  $10^7 - 10^8$  cells/mL, as determined by cell counting 216 (Z2 Coulter Counter, Beckman Coulter<sup>TM</sup>). The amount of algal suspension added, was calculated 217 to give a feeding rate of  $1 \times 10^7$  cells/daphnid/day, which according to reported carbon content of P. 218 subcapitata corresponds to 0.1 mg C/daphnid/day (Halling-Sørensen et al., 1996). Every third day, 219 daphnids were transferred to a clean beakers with fresh medium and the pH and oxygen content of 220 the old media was measured. Endpoints included mortality, molting of carapace, growth, days to 221 first offspring and cumulative number of live and dead offspring. Daphnids were monitored daily to 222 note mortality, molting and the number of live and dead offspring produced (offspring was removed 223 224 and discarded during counting). The body length of daphnids was measured after 21 days from microscope images of the daphnids using QCapture Pro 6.0 image and analysis software. 225

226

#### 227 **2.5 Data treatment and statistical analyses**

228 The measured Cu concentration in suspensions of CuONPs and CuCl<sub>2</sub> was  $95 \pm 10\%$  of the nominal

229 (average and standard deviation), thus nominal Cu concentrations were applied for data treatment.

For AgNO<sub>3</sub> and AgNP suspensions, the average silver recovery was  $69 \pm 21\%$  of the nominal

concentrations. The lower AgNO<sub>3</sub> concentrations (0.2-8  $\mu$ g Ag/L) were bordering the lower limit of

232 the ICP-MS calibration curve (1 µg Ag/L), which may have influenced the recoveries. For AgNPs, incomplete acid digestion of AgNPs prior to ICP-MS analysis may be the cause, but since 100% 233 silver was recovered from the undiluted suspension (as received from the supplier) this must relate 234 to interactions with medium components and/or dilution/transfer steps. Low recoveries of AgNPs in 235 M7 medium is previously reported, even when including sorption to glass walls (Cupi et al., 2015). 236 Due to these analytical uncertainties and because the scope of this study is to compare the effects 237 following continuous and pulse exposure, rather than determining actual toxic levels of silver and 238 copper, the nominal concentrations are applied for data treatment. 239

240

For acute immobility responses, the program TOXCALC<sup>TM</sup> v5.0 (Tidepool Scientific) was used to 241 estimate mean effective concentrations ( $EC_{50}$ ) and 95% confidence intervals by linear regression on 242 probit transformed data using maximum likelihood estimation for the point estimation. 243 The chronic endpoints including body length, number of moltings, days to first live offspring, and 244 total number of live offspring are provided for each replicate mother organism surviving the 21 245 days post-exposure. The chronic data from exposure groups of various concentration and pulse 246 duration were checked for normal distribution (D'Agostin & Pearson normality test) and 247 homogeneity of variances (Bartlett's and Levene's test). In most cases, one or both criteria were not 248 met, even after rank-transformation, and as result one-way analysis of variance (ANOVA) was not 249 applied to compare treatments. Instead, linear regression was applied to identify trends in responses 250 251 with increasing test material concentration (slope  $\neq 0$ , p < 0.05), and to compare if responses of 1, 2 and 3 h pulses differ significantly (slopes of 1, 2 and 3 h data differs, p < 0.05). Deviation from 252 linearity was checked by Runs tests, and was not confirmed for any of the data sets. 253

#### 254 3. Results and discussion

255

#### 256 **3.1 Characterization and stability of test suspensions**

Although the recovery of silver generally was low (i.e. the transfer from stock suspension to tested 257 concentrations in medium), no substantial loss of silver occurred over time during the 24 h aging of 258 259 AgNPs in medium or during the pulse exposures. The silver concentrations measured in AgNP suspensions (200 µg Ag/L) before and after aging, and after pulses were all in the range 53-64%. 260 The samples for ICP-OES analysis were drawn from suspensions without stirring, thus data 261 indicates no substantial sedimentation of AgNPs, and no visible sedimentation or other changes in 262 the appearance of suspensions was observed over 48 hours. According to DLS measurements, the 263 size distribution of AgNPs in medium (200 µg Ag/L) was monodisperse with a single size peak and 264 a polydispersity index (PDI) in the range of 0.14-0.39 for all measurements. The size of AgNPs 265 increased during the 24 h aging step prior to exposure, but then remained relatively stable during 266 267 the following 1-3 hour pulses, see Figure 1A.

268

The zeta potential of the suspensions was at all times within -15 to -18 mV. However, the zeta potential is not necessarily as good an indicator of stability for these citrate stabilized AgNPs, as for strictly electrostatically repulsed NPs. Immediately after suspending AgNPs in modified M7, the dissolved Ag was determined to  $\leq$ 1% of the total measured Ag (Figure 1B). During 24 h aging, the dissolved Ag roughly doubled, and after 26 h (representative of a 2 h pulse exposure), the dissolved fluctuated between 1.5-5% non-dependently of concentration and time (Figure 1B).

275

During CuONP pulse exposures, the measured copper concentration remained within 92-101% of the nominal. However, the distribution of the CuONPs in the test beakers was found to be affected by sedimentation. The recovery of copper (determined as the measured total copper concentration compared to the nominal concentration) was less in samples drawn from the middle of the suspension without stirring, compared to the samples drawn while stirring, and this difference 281 increased with CuONP concentration and pulse duration, see Figure 2A. The sedimentation during pulses was not visible, as suspensions appeared uniform in color, but after 24 and 48 hours of 282 standard testing, sedimentation was visibly detected as a dark layer on the bottom of test beakers, as 283 also reported by others (Adam et al., 2015). Adsorption to containers was also visually detected 284 upon 24 hours as relatively large, dark stains on the inside of plastic flasks used for the aging of 285 CuONP suspensions (data not included). Thus, the impact of sedimentation and adhesion to 286 containers on the exposure concentrations are inevitably greater in a standard 24-48 h toxicity test, 287 compared to the 1-3 hour pulses of CuONPs. The measured zeta potentials of -5 to -13 mV also 288 point to instability of the CuONP suspensions, and this did not change over 3 hours. According to 289 DLS measurements, the CuONP agglomerates formed in modified M7 were micron sized, but the 290 DLS size distribution data was generally of poor quality with a polydispersity index (PDI) of 0.5-291 1.0 and inconsistency between detected size peaks and the z-average sizes. Thus, DLS was deemed 292 inapplicable for providing more detailed information on size distributions over time. The dissolved 293 fraction released from CuONPs as determined by ultracentrifugation was found to be < 0.5% of the 294 total measured Cu and remained rather stable within a 1-3 hour pulse period, but increased to 3.5 295 and 5.5% after 24 and 48 hours, respectively, see figure 2B. The dissolved copper concentration 296 released from the CuONPs was in the range 0.07-72 µg Cu/L (when subtracting the copper nutrient 297 in the medium). This dissolved copper concentration decreased with CuONP concentration (Figure 298 2B), possibly due to the increasing sedimentation with increasing concentration, as also reported by 299 300 others (Baek and An, 2011).

301

302 Overall, the data is in agreement with the expectation that the influence of agglomeration,

dissolution and sedimentation of AgNPs and CuONPs, is minimized during 1-3 hour pulses,

304 compared to standard 24-48 h exposures of *D. magna*, and further for AgNPs, that an aging step is

305 beneficial for maintaining stable exposure conditions, as also suggested for algal toxicity testing

- 306 with AgNPs (Sørensen and Baun, 2015).
- 307

The chemical speciation was estimated by Visual MINTEQ for AgNO<sub>3</sub> and CuCl<sub>2</sub> in modified M7, but not for the tested ENPs as the program is not suited to handle NP specific properties, such as coatings, dissolution, aggregation, surface charge etc. The speciation details for the soluble silver and copper however, provide an indication of the primary chemical species formed from silver and copper in the modified M7 medium. The dominant chemical species are for AgNO<sub>3</sub>: AgCl<sub>aq</sub> (64%), AgCl<sub>2</sub><sup>-</sup> (27%), and Ag<sup>+</sup> (8%), and for CuCl<sub>2</sub>: CuCO<sub>3,aq</sub> (65%), CuEDTA<sup>2-</sup> (13%), CuOH<sup>+</sup> (12%), and Cu<sup>2+</sup> (4%).

315

#### 316 3.2 Acute toxicity of silver and copper to *D. magna* in pulse and continuous exposures Both pulse and continuous exposures to the NPs and the soluble salts of silver were found to be 317 more acutely toxic to *D. magna* than copper, see Figure 3. Mean effective concentrations (EC<sub>50</sub>) and 318 95% confidence intervals for all the curves in Figure 4 are provided in Table 2. For all test 319 materials, the effect level detected 48 h post-exposure from pulse exposures was comparable to the 320 effect level upon 24 h continuous exposures, with EC<sub>50</sub>-values having overlapping 95% confidence 321 intervals. With few exceptions, the 24 and 48 h EC<sub>50</sub>-values from 2 and 3 h pulses were not 322 statistically significant different from those of 24 h continuous exposures, as evidenced by the 323 overlapping 95% confidence intervals. Generally, fewer immobile organisms were observed 324 immediately after the pulse exposures (0 h post-exposure) than after 24 and 48 hours post-exposure 325 observation. This indicates that the organisms were indeed affected by the short-term pulses, 326 327 although this effect on mobility could not be observed until 24 hours later. The slightly lower acute toxicity of CuONP pulses compared to the continuous exposure indicates a somewhat slower 328 toxicokinetics and/or toxicodynamics than is the case for AgNPs. Ionic silver $(Ag^+)$ is known to 329 inhibit the active $Na^+$ uptake by blocking the $N^+$ , $K^+$ -ATPase, a process shown to occur within 2 330 hours and be complete within 12 h in Daphnia magna (Bianchini and Wood, 2003). The acute 331 toxicity of copper may involve a similar physiological mechanism (Bianchini and Wood, 2003), but 332 also the redox cycling of copper and following generation of reactive oxygen species has been 333 reported as mechanisms involved in cellular toxicity of copper (Thit et al., 2015). In contrast to 334

335 silver, copper is an essential metal for cells, and may explain why higher concentrations and/or longer exposure time is needed for toxicity to occur. Moreover, the differences in silver and copper 336 toxicity may relate to the bioavailability differing due to chemical speciation in the medium and/or 337 affinity for the binding site. According to the chemical speciation modeling, the percentage of free 338 ionic  $Ag^+$  (8%) is twice that of  $Cu^{2+}$  (4%) in the modified M7 medium. This correlates well with the 339 higher toxicity of AgNO<sub>3</sub> and AgNPs, although the contribution to toxicity from other chemical 340 species such as CuCO<sub>3</sub>, CuOH<sup>+</sup> and AgCl<sub>ag</sub> cannot be ruled out (De Schamphelaere and Janssen, 341 2004; SCENIHR, 2014). Rapid toxicokinetics and toxicodynamics would explain why a few hours 342 pulse exposure to AgNO<sub>3</sub> and CuCl<sub>2</sub> is sufficient to cause the same level of effects as could be 343 observed in the standard 48 h acute toxicity tests with continuous exposure. This trend was also 344 observed for the AgNPs and CuONPs, indicating that the dissolved fraction is governing toxicity. 345 The EC<sub>50</sub>-values of CuCl<sub>2</sub> were about 100 times lower than for CuONPs and for AgNO<sub>3</sub> the values 346 were about 20 times lower than for AgNPs. The aging step introduced prior to the testing of AgNPs 347 may have influenced this difference as the AgNP suspension had a head start in terms of dissolution 348 compared to tests with freshly prepared CuONP suspensions. However, in tests with CuONPs rapid 349 sedimentation occurred in the test suspensions. This decreased the availability of CuONPs to the 350 daphnids even within the short pulse exposures. For all tested materials the 24 and 48 h  $EC_{50}$ -values 351 decreased with increased pulse duration although not statistically significant (p > 0.05). Thus, short-352 term (1-3 hour) pulse tests were found to be sufficient to disclose the acute toxicity of CuCl<sub>2</sub>, 353 354 CuONPs, AgNO<sub>3</sub> and AgNPs.

355

# 356 **3.3** Chronic effects of silver and copper pulses on *D. magna* mortality, body length and molting

In the 21 days post-exposure period following 1, 2 and 3 hour pulse exposures of the test materials, the mortality observed in the exposed groups was neither concentration nor pulse-duration dependent. (Figure S1). According to the OECD guideline 211 (OECD, 2012), one of the validity criteria is a control mortality of  $\leq 20\%$ . In three of the nine tests, a control mortality of 40-50% 362 occurred (control groups for 1 and 2 hour AgNO<sub>3</sub> pulse, and 3 hour CuONPs pulse, respectively). 363 For the CuONP pulse, this was not reflected by an overall high level of mortality in the exposed 364 groups ( $\leq 20\%$ ), and the results of this test are therefore considered valid. For the AgNO<sub>3</sub> tests, high 365 mortalities generally occurred across exposure groups, however the high mortalities in the control 366 groups hamper the interpretation of these mortality results.

367

The measured body length of surviving mother organisms after 21 days post-exposure did not change as a function of concentration or pulse-duration for any of the test materials (Figure S2). Linear regression slopes were not statistically significant different for data sets obtained after 1, 2 and 3 h pulse exposures (p < 0.05). These data sets s were pooled and the slopes of linear regression curves of body lengths versus concentration were not statistically significant different from zero for any of the test materials (p > 0.05).

374

375 The number of moltings per surviving mother organism was not affected by the pulse-duration,

376 however the linear regressions on pooled data indicate a slight decrease in the number of moltings

377 with concentration for  $CuCl_2$  and CuONPs, but not for AgNO<sub>3</sub> and AgNPs (Figure S2).

378

#### 379 **3.4 Effects of silver and copper pulses on reproduction of** *D. magna*

For AgNPs and AgNO<sub>3</sub>, the time to first live offspring did not change with silver concentration or 380 381 pulse duration (Figure 4A and B). The number of offspring however, appear to increase slightly with silver concentration (Figure 4E and F), although only statistically significant for the 3 hours 382 pulse exposure to AgNPs (p < 0.05). A stimulating effect on the number of offspring has previously 383 been reported for the same type of AgNPs at 10 µg Ag/L in a static renewal setup (Mackevica et al., 384 2015) and for other citrate-coated AgNPs at 2 µg Ag/L (Pokhrel and Dubey, 2012). This hormetic 385 effect of silver could result from the antibacterial effects of silver causing better conditions for the 386 test organism as also suggested by Mackevica et al. (2015). Other studies have found detrimental 387 effects of AgNPs on the survival, growth and/or reproduction of D. magna at 10-50 µg Ag/L 388

389 (Mackevica et al., 2015; Zhao and Wang, 2011). For AgNO<sub>3</sub>, no effects on growth, time to first brood or number of offspring were observed for continuous exposures in concentrations up to 1.6 390 ug Ag/L during 21 days, but mortality was shown to be affected at 1.6 ug Ag/L (Mackevica et al., 391 2015; Zhao and Wang, 2011). Overall, the results of the present pulse tests together with published 392 data from tests with continuous exposures suggest that 1-3 h pulses of AgNO<sub>3</sub> and AgNPs, 393 respectively, do not induce chronic effects to the same extent as 21 d continuous exposure.

395

394

For CuONP pulses, the data indicate slightly longer time to first offspring for 3 h pulse exposures 396 and pronounced decreases in the number of offspring with copper concentration for all pulse 397 durations (Figure 4C and G). Similarly, for CuCl<sub>2</sub> the number of offspring decreased after 2 and 3 h 398 pulses, whereas the time to offspring was unaffected (or even reduced after 1 h pulse exposure) 399 (Figure 4D and H). Continuous exposure tests (i.e., static renewal tests) with D. magna to the same 400 type of CuONPs and CuCl<sub>2</sub> have been reported to decrease the number of offspring with 401 concentration in the ranges: 0.037-1.4 mg Cu/L (CuONPs) and 0.004-0.060 mg Cu/L (CuCl<sub>2</sub>) 402 403 (Adam et al., 2015). These ranges are comparable to observation made in the present study (Figure 4), however the number of offspring was far more affected in the continuous exposure setup than in 404 our pulse exposure tests, with a 20% reduction or more in the number of offspring at the highest 405 exposure concentrations (Adam et al., 2015). Moreover, a significant decrease in the body lengths 406 was found in the same study at 1.4 and 0.03 mg Cu/L for CuONPs and CuCl<sub>2</sub>, respectively (Adam 407 408 et al., 2015). Another study with continuous exposure (daily renewal) found CuONPs to significantly inhibit both growth and reproduction, at very low concentrations of 0.002 mg Cu/L, 409 but not the time to first brood (Zhao et al., 2012). Thus, a short-term pulse of soluble copper or 410 CuONPs may induce chronic effects, although at higher concentrations than for continuous 411 exposure. 412

413 To elucidate whether the effect of CuONP pulses on the number of offspring was caused by dissolved copper, the exposure concentrations of CuONPs in Figure 4G was converted into 414 dissolved copper concentrations based on the dissolution measurements, and compared with the 415 data for CuCl<sub>2</sub> in Figure 4H. Since the linear regressions of the number of offspring as a function of 416 the dissolved copper concentrations for 1, 2 and 3 h CuONPs pulses were not statistically 417 418 significant different with respect to slope and constant term, these data were pooled to simplify the comparison. For CuCl<sub>2</sub>, pooling of data from 1, 2 and 3 h pulses was not possible due to different 419 slopes and/or elevations. Instead, the data with the steepest slope was used for the comparison (2 h 420 pulse). Comparing the two datasets and the linear regressions (Figure S3), the effects of dissolved 421 Cu from CuONPs were more pronounced than for CuCl<sub>2</sub>, indicating a nano-specific effect on 422 toxicity or bioavailability. This could relate to the physical dimension of NPs, causing them to 423 accumulate in the gut of the daphnids as result of their filter-feeding behavior. Accumulation in the 424 gut may cause CuONPs to dissolve more readily as result of the environment in the gut, as 425 suggested for AgNPs (Gaiser et al., 2011), but remaining CuO particles in the gut region of the 426 daphnids may also increase the internal exposure beyond the pulse duration. In line with this, higher 427 body burdens of copper have been measured in daphnids exposed to CuONPs, compared to CuCl<sub>2</sub> 428 (Adam et al., 2015), although the effects on reproduction in that study were similar for CuCl<sub>2</sub> and 429 430 CuONPs when the results were expressed as dissolved copper.

431

432 The OECD guideline no. 211 includes a validity criteria of a mean number of  $\geq$  60 live offspring produced per surviving mother organism in unexposed control groups after 21 days (OECD, 2012). 433 This criterion was met for four of the twelve tests, short by a few in four tests (1 and 3 h CuCl<sub>2</sub>, 1 h 434 and 3 h pulse AgNO<sub>3</sub>) and short by more (a total of 37-49 offspring per daphnid) in the three tests 435 with AgNP pulses. The offspring production was however in the same range as for the pre-culture 436 and because there was a positive effect or no effect on number of offspring in the tests with lowest 437 control reproduction (AgNPs and AgNO<sub>3</sub> pulses), this lower production is not assumed to indicate 438 lower fitness of the organisms and thereby have influenced the test outcome. 439

Overall, the concentrations found to cause immobilization in *D. magna* of both silver forms (AgNO<sub>3</sub> and AgNPs) and CuCl<sub>2</sub> after 2-3 h pulse exposures were similar to those found for 24 h continuous exposure. For CuONPs the effects of pulses on *D. magna* mobility were slightly lower than those observed after continuous exposure. In contrast, the reproductive effects were most pronounced for CuONPs, whereas exposure to AgNPs in sub-lethal concentrations seemed to slightly stimulate reproduction.

447

The 1, 2, and 3 h pulses of AgNO<sub>3</sub> and AgNPs caused acute effects at concentration levels 448 comparable to what has been reported in the literature for 48 h continuous exposure. Our results 449 showed that if a few hours of exposure to silver do not cause lethality in D. magna, then neither 450 delayed mortality nor effects on molting, body length and reproduction are likely to occur. While 451 the toxic mode of action governing acute toxicity of silver is well-known, the mechanism behind 452 reduced offspring production is less clear and may involve transfer of egg yolk protein or simply a 453 diversion of metabolic energy away from reproduction, towards metal detoxification (Wood et al., 454 455 2002).

#### 456 4. Conclusion

457

This study aimed to investigate the applicability of 1-3 h pulse exposure for stabilizing exposures 458 and disclosing acute and chronic effects of AgNPs, CuONPs, AgNO<sub>3</sub> and CuCl<sub>2</sub> in *D. magna*. 459 Overall, the impact of dissolution, agglomeration and sedimentation on the stability of ENPs in 460 daphnia test medium was less during 1-3 h, compared to the 24-48 h representative of a standard 461 acute immobility test. The level of immobility observed for D. magna 48 h after a short-term pulse 462 exposure (1, 2, and 3 h) to the test materials was generally comparable to the effect levels observed 463 after 24 h continuous exposure. For CuONPs the effects of pulse exposures were slightly lower than 464 for continuous exposure, although not statistically. With regards to chronic effects, the 1-3 h pulses 465 466 impacted mortality, body length and reproduction less than what is reported in literature for chronic tests with continuous exposures to the same test materials. 467

468

Pulse exposure tests with *D. magna* may serve for acute toxicity screening of engineered
nanoparticles as they represent environmentally relevant exposure scenarios and enable more stable
exposure scenarios to be maintained during the incubation in test medium, than standard continuous
exposure tests. Applicability was demonstrated in terms of acute immobility for AgNPs and
CuONPs, but pulse exposure tests may very likely be applicable for acute toxicity testing of other

474 very toxic and partly dissolving metal NPs.

475

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#### 614 Tables

#### 615

**Table 1.** Nominal exposure concentration ranges in toxicity tests with *D. magna*.

Toxicity test	AgNO <sub>3</sub>	AgNPs	CuCl <sub>2</sub>	CuONPs
	μg Ag/L		mg Cu/L	
Acute effects (24-48 h continuous exposure)	0.38-6.0	16-80	0.01-0.80	0.25-4.0
Acute effects of pulse (1, 2, 3 hour pulses)	0.50-8.0	25-200	0.03-0.80	1.3-20
Chronic effects of pulse (1, 2, 3 hour pulses)	0.20-2.0	10-50	0.01-0.05	0.20-3.2

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- **Table 2.**  $EC_{50}$ -values with 95% confidence intervals in brackets for acute immobilization responses occurring after 24 and 48 hours standard continuous exposures, and after 0, 24 and 48 hours postexposure following 1, 2 and 3 hour pulses to AgNPs, AgNO<sub>3</sub>, CuONPs and CuCl<sub>2</sub>.
- 622

Exposure type	AgNPs	AgNO <sub>3</sub>	CuONPs	CuCl <sub>2</sub>
Exposure type	μg Ag/L	µg Ag/L	mg Cu/L	mg Cu/L
Continuous				
EC <sub>50,24h</sub> EC <sub>50,48h</sub>	130 [120 ; 150] n.d.	4.5 [4.0 ; 5.2] 4.0 [n.e.]	6.5 [3.7 ; 46] 5.2 [2.9 ; 25]	0.10 [0.080 ; 0.13] 0.05* [n.e.]
1 h pulse				
EC <sub>50,0h</sub> post-exposure EC <sub>50,24h</sub> post-exposure EC <sub>50,48h</sub> post-exposure	290 [200 ; 9700] 130 [110 ; 150] 110 [88 ; 140]	8* [n.e.] 6.2 [n.e.] 5.1 [4.3 ; 6.5]	130 [31;4.9×10 <sup>8</sup> ] 280 [n.e.] 42 [15 ; 89000]	1.1 [0.70 ; 4.3] 0.27 [0.21 ; 0.34] 0.22 [0.036 ; 0.75]
2 h pulse				
EC50,0h post-exposure EC50,24h post-exposure EC50,48h post-exposure	150 [130 ; 200] 120 [n.e.] 110 [n.e.]	> 8* [n.e.] 6.2 [5.2 ; 8.2] 4.6 [3.7 ; 6.0]	260 [n.e.] 33 [15 ; 520] 19 [12 ; 66]	0.34 [n.e.] 0.17 [0.12 ; 0.22] 0.14 [0.031 ; 0.42]
3 h pulse				
EC <sub>50,0h</sub> post-exposure EC <sub>50,24h</sub> post-exposure EC <sub>50,48h</sub> post-exposure	110 [90 ; 130] 110 [92 ; 130] 110 [87 ; 120]	9.8 [7.0 ; 27] 6.4 [5.2 ; 9.1] 4.3 [3.1 ; 7.0]	43 [17 ; 4800] 22 [12 ; 110] 17 [11 ; 47]	0.27 [0.22 ; 0.34] 0.14 [n.e.] 0.12* [n.e.]

623 n.d. Not determined.

n.e. Not estimated. Estimation of confidence interval is not possible from data.

\*Value determined from linear connection of data points (estimation by TOXCALC not possible).





**Figure 1.** Agglomeration and dissolution of AgNPs in modified M7. A) Mean size of the single size peak detected by DLS for AgNPs in modified M7 (of nominal concentration 200  $\mu$ g Ag/L) immediately upon preparation (0 h), after aging (24 h), and after pulse exposure (1, 2 and 3 h, respectively). B) Dissolved silver measured in the supernatant of ultracentrifuged AgNP suspensions (of nominal concentrations 10, 50 and 200  $\mu$ g Ag/L) over time, as % of the total measured Ag concentrations at time 0, 24 and 48 h, respectively.

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Figure 2. Sedimentation and dissolution of CuONPs in modified M7. A) Measured copper concentration as percent of nominal in CuONP suspensions (1.3 and 20 mg Cu/L) after ended pulse exposures (1, 2 and 3 h) sampled first without stirring from center of suspensions, and then sampled during stirring, yielding the suspended and total copper, respectively. B) Fraction of dissolved copper measured in the supernatant of ultracentrifuged CuONP suspensions (of nominal concentrations 0.2, 2 and 20 mg Cu/L) determined 0, 1, 2, 3, 24 and 48 hours after suspending the CuONPs (shown as percent of the total measured copper concentration at 0 h). All measured Cu concentrations are corrected for the Cu nutrient of the medium.







**Figure 4.** Reproduction data for *D. magna* in the 21 days post-exposure observation period after 1, 2, and 3 h pulse exposure to AgNPs,  $AgNO_3$ , CuONPs and CuCl<sub>2</sub> (columns 1-4). Data are observations made over the 21 days period for each surviving mother organism, along with linear regressions for 1, 2 and 3 h pulses, respectively. Row 1: Days to first live offspring. Row 2: Total number of live offspring produced in the postexposure period per surviving mother organism. Parameters and statistics for linear regressions are provided in Table S2.