



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

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1 **Title**

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

3

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ABSTRACT

18
19 Aquatic toxicity testing of nanoparticles (NPs) is challenged by their dynamic behavior in test
20 suspensions. The resulting difficulties in controlling and characterizing exposure concentrations are
21 detrimental to the generation of concentration-response data needed for hazard identification of
22 NPs. This study explores the applicability of short-term (1, 2 and 3 h) pulse exposures as means to
23 keep the exposure stable and at the same time disclose acute and chronic effects of AgNPs and
24 CuONPs in *D. magna*. Dissolution, agglomeration and sedimentation were found to have less
25 influence on exposure concentrations during 1-3 h pulses than for 24-48 h continuous exposures.
26 For AgNPs, preparation of test suspensions in medium 24 h before toxicity testing (aging) increased
27 stability during the short-term pulses. In pulse tests, organisms were exposed to the test materials
28 AgNPs and CuONPs for 1, 2 and 3 h, and afterwards transferred to clean medium and observed for
29 48 h (post-exposure period) for acute effects and for 21 d for chronic effects. AgNO₃ and CuCl₂
30 were used as reference materials for dissolved silver and copper, respectively. For all test materials,
31 a 3 h pulse caused comparable immobility in *D. magna* (observed after 48 h post-exposure) as 24 h
32 continuous exposure, as evidenced by overlapping 95% confidence intervals of EC₅₀-values. In the
33 21 d post-exposure period, no trends in mortality or body length were identified. AgNPs and
34 AgNO₃ pulses had no effect on the number of moltings, days to first live offspring or cumulated
35 number of offspring, but the number of offspring increased for AgNPs (3 h pulse only). In contrast,
36 CuONPs and CuCl₂ pulses decreased the number of moltings and offspring, and for CuONPs the
37 time to first live offspring was prolonged. After CuONP exposures, the offspring production
38 decreased more with increasing concentrations than for CuCl₂ exposures when taking the measured
39 dissolved copper into account. This indicates a nanoparticle-specific effect for CuONPs, possible
40 related CuONPs accumulated in the gut of *D. magna* during the pulse exposure. Pulse exposure is
41 an environmentally relevant exposure scenario for NPs, which for AgNPs and CuONPs enables
42 more stable exposures and cause acute immobility of *D. magna* comparable to continuous 24 h
43 exposures. Pulse exposure is likely relevant and applicable for other toxic and dissolving metal NPs,
44 but this requires further research.

45

46 **Keywords**

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

48 **1. Introduction**

49

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

66

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

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

85
86 Short-term exposures in *D. magna* identified as “pulse” or “intermittent” exposures have been
87 applied for toxicity testing of both organic and inorganic substances, with the effects being
88 monitored immediately after or during a post-exposure period in clean media after the pulse
89 (Andersen et al., 2006; Hoang and Klaine, 2008; Trac et al., 2015). Pulse toxicity tests have been
90 applied mainly to simulate a more environmentally realistic and relevant exposure scenario, as
91 many chemical pollutants are discharged into aquatic environments as “pulses” resulting from e.g.
92 surface runoff after rain events, overflow of wastewater treatment plants, agrochemicals and
93 veterinary pharmaceuticals from agriculture (e.g. Handy, 1994; Hommen et al., 2010). Furthermore,
94 agrochemicals with a short half-life in the environment have also been suggested to exhibit pulse-
95 like exposures (Reinert et al., 2002). Analogously, ENPs are also expected to rapidly transform
96 from their pristine form, due to for example dissolution, agglomeration, sedimentation, and coating
97 alterations once discharged into the environment (Lowry et al., 2014) or even when added to
98 simplified media in laboratory toxicity tests (Petersen et al., 2014). A study of stream mesocosms
99 has found the fate of cerium oxide ENPs to differ for press and pulse exposures and the authors
100 recommend environmental risk assessment of ENPs to address the implications of exposure
101 duration (Baker et al., 2016). To date, several studies have investigated the chronic effects in

102 crustaceans exposed to ENPs of silver (Blinova et al., 2013; Gaiser et al., 2011; Mackevica et al.,
103 2015; Pokhrel and Dubey, 2012; Seitz et al., 2015; Zhao and Wang, 2011), TiO₂ (Seitz et al., 2013),
104 ZnO (Adam et al., 2014; Zhao et al., 2012), and CuO (Adam et al., 2015; Rossetto et al., 2014;
105 Zhao et al., 2012). These studies focus on a variety of influencing factors such as feeding
106 conditions, exposure routes, exposure scenarios (semi-static vs. flow-through), mixture effects, ENP
107 characteristics (e.g. size, agglomeration, coating), environmental factors (e.g. pH, media, dissolved
108 organic matter) and effects of ENPs vs. released ions. However, to our knowledge, the influence of
109 exposure duration, in terms of a short-term NP pulse, and the following acute and chronic effect to
110 *D. magna* remains unexamined.

111

112 The aim of this study is to explore the applicability of a short-term pulse exposure to reveal acute
113 and chronic effects of Ag and CuONPs to *D. magna*. We exposed neonate *D. magna* to 1, 2 and 3 h
114 pulse exposures to AgNPs, CuONPs, silver nitrate (AgNO₃) and copper chloride (CuCl₂) and
115 studied the acute (immobility) and chronic effects (mortality, body length, molting and
116 reproduction) occurring within 48 h and 21 d post-exposure periods, respectively. The obtained
117 responses were then compared to those of continuous exposures in acute and chronic tests with the
118 same materials. Pulsed exposure presents an environmentally relevant exposure scenario and may
119 also facilitate more stable exposure conditions for ENPs during testing. As the transformation
120 processes of suspended ENPs are time-dependent, we expected less impact of such processes during
121 a short-term pulse, than during 24 or 48 h continuous exposure applied for standard acute tests.
122 Therefore, the stability of the test suspensions in terms of agglomeration, dissolution and
123 sedimentation of AgNPs and CuONPs was monitored over the course of the pulse (1-3 h) and
124 continuous (48 h) exposures.

125 **2. Materials and Methods**

126

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

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

138

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

152

153 **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 HNO₃ to a concentration of 17% (AgNPs) and 6.2% (CuONPs) and digested for 24
159 hours at 20 °C in the dark, then diluted with de-ionized water and kept in the dark at 4 °C until
160 analysis.

161

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

168

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

174

175 The concentration of dissolved Ag and Cu from NP suspensions in medium was determined by
176 ultracentrifugation (Beckman L8-60M). Briefly, suspensions of AgNP (10, 50 and 200 µg Ag/L
177 nominal) and CuONP (0.2, 2 and 20 mg Cu/L nominal) were prepared in medium and kept in the
178 dark at 20 °C, similar to the exposure conditions of toxicity testing and aging. Duplicate samples of

179 10 mL were drawn from each suspension at times reflecting the pulse and continuous exposure
180 durations (and for AgNPs also the aging step before testing); For CuONPs at 0, 1, 2, 3, 24 and 48
181 hours and for AgNPs at 0, 24, 25, 26, 27, and 48 h. The samples were centrifuged for 30 min
182 (AgNPs) and 45 min (CuONPs) at 30000 rpm ($\sim 68000 \times g$) to ensure settling of particles according
183 to their sizes (based on calculations derived from Stoke's law and material density). The
184 supernatant (5 mL) was collected, digested with HNO₃ and the Ag and Cu concentration determined
185 by ICP-MS.

186 Speciation of Ag and Cu from AgNO₃ and CuCl₂ in modified M7 was estimated by the chemical
187 speciation modeling program Visual MINTEQ 3.0 (Gustafsson, 2010), assuming equilibrium with
188 atmospheric CO₂.

189

190 **2.3 Acute toxicity tests**

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

203

204 **2.4 Chronic toxicity tests**

205 The chronic toxicity following a single pulse exposure to the test materials was investigated over a
206 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 ± 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.

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

226

227 **2.5 Data treatment and statistical analyses**

228 The measured Cu concentration in suspensions of CuONPs and CuCl₂ was $95 \pm 10\%$ of the nominal
229 (average and standard deviation), thus nominal Cu concentrations were applied for data treatment.

230 For AgNO₃ and AgNP suspensions, the average silver recovery was $69 \pm 21\%$ of the nominal
231 concentrations. The lower AgNO₃ concentrations (0.2-8 µg Ag/L) were bordering the lower limit of

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

240

241 For acute immobility responses, the program TOXCALCTM v5.0 (Tidepool Scientific) was used to
242 estimate mean effective concentrations (EC_{50}) and 95% confidence intervals by linear regression on
243 probit transformed data using maximum likelihood estimation for the point estimation.

244 The chronic endpoints including body length, number of moltings, days to first live offspring, and
245 total number of live offspring are provided for each replicate mother organism surviving the 21
246 days post-exposure. The chronic data from exposure groups of various concentration and pulse
247 duration were checked for normal distribution (D'Agostin & Pearson normality test) and
248 homogeneity of variances (Bartlett's and Levene's test). In most cases, one or both criteria were not
249 met, even after rank-transformation, and as result one-way analysis of variance (ANOVA) was not
250 applied to compare treatments. Instead, linear regression was applied to identify trends in responses
251 with increasing test material concentration (slope $\neq 0$, $p < 0.05$), and to compare if responses of 1, 2
252 and 3 h pulses differ significantly (slopes of 1, 2 and 3 h data differs, $p < 0.05$). Deviation from
253 linearity was checked by Runs tests, and was not confirmed for any of the data sets.

254 **3. Results and discussion**

255

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

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

268

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

275

276 During CuONP pulse exposures, the measured copper concentration remained within 92-101% of
277 the nominal. However, the distribution of the CuONPs in the test beakers was found to be affected
278 by sedimentation. The recovery of copper (determined as the measured total copper concentration
279 compared to the nominal concentration) was less in samples drawn from the middle of the
280 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
282 pulses was not visible, as suspensions appeared uniform in color, but after 24 and 48 hours of
283 standard testing, sedimentation was visibly detected as a dark layer on the bottom of test beakers, as
284 also reported by others (Adam et al., 2015). Adsorption to containers was also visually detected
285 upon 24 hours as relatively large, dark stains on the inside of plastic flasks used for the aging of
286 CuONP suspensions (data not included). Thus, the impact of sedimentation and adhesion to
287 containers on the exposure concentrations are inevitably greater in a standard 24-48 h toxicity test,
288 compared to the 1-3 hour pulses of CuONPs. The measured zeta potentials of -5 to -13 mV also
289 point to instability of the CuONP suspensions, and this did not change over 3 hours. According to
290 DLS measurements, the CuONP agglomerates formed in modified M7 were micron sized, but the
291 DLS size distribution data was generally of poor quality with a polydispersity index (PDI) of 0.5-
292 1.0 and inconsistency between detected size peaks and the z-average sizes. Thus, DLS was deemed
293 inapplicable for providing more detailed information on size distributions over time. The dissolved
294 fraction released from CuONPs as determined by ultracentrifugation was found to be < 0.5% of the
295 total measured Cu and remained rather stable within a 1-3 hour pulse period, but increased to 3.5
296 and 5.5% after 24 and 48 hours, respectively, see figure 2B. The dissolved copper concentration
297 released from the CuONPs was in the range 0.07-72 µg Cu/L (when subtracting the copper nutrient
298 in the medium). This dissolved copper concentration decreased with CuONP concentration (Figure
299 2B), possibly due to the increasing sedimentation with increasing concentration, as also reported by
300 others (Baek and An, 2011).

301

302 Overall, the data is in agreement with the expectation that the influence of agglomeration,
303 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

308 The chemical speciation was estimated by Visual MINTEQ for AgNO₃ and CuCl₂ in modified M7,
309 but not for the tested ENPs as the program is not suited to handle NP specific properties, such as
310 coatings, dissolution, aggregation, surface charge etc. The speciation details for the soluble silver
311 and copper however, provide an indication of the primary chemical species formed from silver and
312 copper in the modified M7 medium. The dominant chemical species are for AgNO₃: AgCl_{aq} (64%),
313 AgCl₂⁻ (27%), and Ag⁺ (8%), and for CuCl₂: CuCO_{3, aq} (65%), CuEDTA²⁻ (13%), CuOH⁺ (12%),
314 and Cu²⁺ (4%).

315

316 **3.2 Acute toxicity of silver and copper to *D. magna* in pulse and continuous exposures**

317 Both pulse and continuous exposures to the NPs and the soluble salts of silver were found to be
318 more acutely toxic to *D. magna* than copper, see Figure 3. Mean effective concentrations (EC₅₀) and
319 95% confidence intervals for all the curves in Figure 4 are provided in Table 2. For all test
320 materials, the effect level detected 48 h post-exposure from pulse exposures was comparable to the
321 effect level upon 24 h continuous exposures, with EC₅₀-values having overlapping 95% confidence
322 intervals. With few exceptions, the 24 and 48 h EC₅₀-values from 2 and 3 h pulses were not
323 statistically significant different from those of 24 h continuous exposures, as evidenced by the
324 overlapping 95% confidence intervals. Generally, fewer immobile organisms were observed
325 immediately after the pulse exposures (0 h post-exposure) than after 24 and 48 hours post-exposure
326 observation. This indicates that the organisms were indeed affected by the short-term pulses,
327 although this effect on mobility could not be observed until 24 hours later. The slightly lower acute
328 toxicity of CuONP pulses compared to the continuous exposure indicates a somewhat slower
329 toxicokinetics and/or toxicodynamics than is the case for AgNPs. Ionic silver (Ag⁺) is known to
330 inhibit the active Na⁺ uptake by blocking the N⁺, K⁺-ATPase, a process shown to occur within 2
331 hours and be complete within 12 h in *Daphnia magna* (Bianchini and Wood, 2003). The acute
332 toxicity of copper may involve a similar physiological mechanism (Bianchini and Wood, 2003), but
333 also the redox cycling of copper and following generation of reactive oxygen species has been
334 reported as mechanisms involved in cellular toxicity of copper (Thit et al., 2015). In contrast to

335 silver, copper is an essential metal for cells, and may explain why higher concentrations and/or
336 longer exposure time is needed for toxicity to occur. Moreover, the differences in silver and copper
337 toxicity may relate to the bioavailability differing due to chemical speciation in the medium and/or
338 affinity for the binding site. According to the chemical speciation modeling, the percentage of free
339 ionic Ag^+ (8%) is twice that of Cu^{2+} (4%) in the modified M7 medium. This correlates well with the
340 higher toxicity of AgNO_3 and AgNPs, although the contribution to toxicity from other chemical
341 species such as CuCO_3 , CuOH^+ and AgCl_{aq} cannot be ruled out (De Schamphelaere and Janssen,
342 2004; SCENIHR, 2014). Rapid toxicokinetics and toxicodynamics would explain why a few hours
343 pulse exposure to AgNO_3 and CuCl_2 is sufficient to cause the same level of effects as could be
344 observed in the standard 48 h acute toxicity tests with continuous exposure. This trend was also
345 observed for the AgNPs and CuONPs, indicating that the dissolved fraction is governing toxicity.
346 The EC_{50} -values of CuCl_2 were about 100 times lower than for CuONPs and for AgNO_3 the values
347 were about 20 times lower than for AgNPs. The aging step introduced prior to the testing of AgNPs
348 may have influenced this difference as the AgNP suspension had a head start in terms of dissolution
349 compared to tests with freshly prepared CuONP suspensions. However, in tests with CuONPs rapid
350 sedimentation occurred in the test suspensions. This decreased the availability of CuONPs to the
351 daphnids even within the short pulse exposures. For all tested materials the 24 and 48 h EC_{50} -values
352 decreased with increased pulse duration although not statistically significant ($p > 0.05$). Thus, short-
353 term (1-3 hour) pulse tests were found to be sufficient to disclose the acute toxicity of CuCl_2 ,
354 CuONPs, AgNO_3 and AgNPs.

355

356 **3.3 Chronic effects of silver and copper pulses on *D. magna* mortality, body length and** 357 **molting**

358 In the 21 days post-exposure period following 1, 2 and 3 hour pulse exposures of the test materials,
359 the mortality observed in the exposed groups was neither concentration nor pulse-duration
360 dependent. (Figure S1). According to the OECD guideline 211 (OECD, 2012), one of the validity
361 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 AgNO₃ 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 AgNO₃ 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

368 The measured body length of surviving mother organisms after 21 days post-exposure did not
369 change as a function of concentration or pulse-duration for any of the test materials (Figure S2).
370 Linear regression slopes were not statistically significant different for data sets obtained after 1, 2
371 and 3 h pulse exposures ($p < 0.05$). These data sets were pooled and the slopes of linear regression
372 curves of body lengths versus concentration were not statistically significant different from zero for
373 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 CuCl₂ and CuONPs, but not for AgNO₃ and AgNPs (Figure S2).

378

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

380 For AgNPs and AgNO₃, the time to first live offspring did not change with silver concentration or
381 pulse duration (Figure 4A and B). The number of offspring however, appear to increase slightly
382 with silver concentration (Figure 4E and F), although only statistically significant for the 3 hours
383 pulse exposure to AgNPs ($p < 0.05$). A stimulating effect on the number of offspring has previously
384 been reported for the same type of AgNPs at 10 $\mu\text{g Ag/L}$ in a static renewal setup (Mackevica et al.,
385 2015) and for other citrate-coated AgNPs at 2 $\mu\text{g Ag/L}$ (Pokhrel and Dubey, 2012). This hormetic
386 effect of silver could result from the antibacterial effects of silver causing better conditions for the
387 test organism as also suggested by Mackevica et al. (2015). Other studies have found detrimental
388 effects of AgNPs on the survival, growth and/or reproduction of *D. magna* at 10-50 $\mu\text{g Ag/L}$

389 (Mackevica et al., 2015; Zhao and Wang, 2011). For AgNO₃, no effects on growth, time to first
390 brood or number of offspring were observed for continuous exposures in concentrations up to 1.6
391 µg Ag/L during 21 days, but mortality was shown to be affected at 1.6 µg Ag/L (Mackevica et al.,
392 2015; Zhao and Wang, 2011). Overall, the results of the present pulse tests together with published
393 data from tests with continuous exposures suggest that 1-3 h pulses of AgNO₃ and AgNPs,
394 respectively, do not induce chronic effects to the same extent as 21 d continuous exposure.
395

396 For CuONP pulses, the data indicate slightly longer time to first offspring for 3 h pulse exposures
397 and pronounced decreases in the number of offspring with copper concentration for all pulse
398 durations (Figure 4C and G). Similarly, for CuCl₂ the number of offspring decreased after 2 and 3 h
399 pulses, whereas the time to offspring was unaffected (or even reduced after 1 h pulse exposure)
400 (Figure 4D and H). Continuous exposure tests (i.e., static renewal tests) with *D. magna* to the same
401 type of CuONPs and CuCl₂ have been reported to decrease the number of offspring with
402 concentration in the ranges: 0.037-1.4 mg Cu/L (CuONPs) and 0.004-0.060 mg Cu/L (CuCl₂)
403 (Adam et al., 2015). These ranges are comparable to observation made in the present study (Figure
404 4), however the number of offspring was far more affected in the continuous exposure setup than in
405 our pulse exposure tests, with a 20% reduction or more in the number of offspring at the highest
406 exposure concentrations (Adam et al., 2015). Moreover, a significant decrease in the body lengths
407 was found in the same study at 1.4 and 0.03 mg Cu/L for CuONPs and CuCl₂, respectively (Adam
408 et al., 2015). Another study with continuous exposure (daily renewal) found CuONPs to
409 significantly inhibit both growth and reproduction, at very low concentrations of 0.002 mg Cu/L,
410 but not the time to first brood (Zhao et al., 2012). Thus, a short-term pulse of soluble copper or
411 CuONPs may induce chronic effects, although at higher concentrations than for continuous
412 exposure.

413 To elucidate whether the effect of CuONP pulses on the number of offspring was caused by
414 dissolved copper, the exposure concentrations of CuONPs in Figure 4G was converted into
415 dissolved copper concentrations based on the dissolution measurements, and compared with the
416 data for CuCl₂ in Figure 4H. Since the linear regressions of the number of offspring as a function of
417 the dissolved copper concentrations for 1, 2 and 3 h CuONPs pulses were not statistically
418 significant different with respect to slope and constant term, these data were pooled to simplify the
419 comparison. For CuCl₂, pooling of data from 1, 2 and 3 h pulses was not possible due to different
420 slopes and/or elevations. Instead, the data with the steepest slope was used for the comparison (2 h
421 pulse). Comparing the two datasets and the linear regressions (Figure S3), the effects of dissolved
422 Cu from CuONPs were more pronounced than for CuCl₂, indicating a nano-specific effect on
423 toxicity or bioavailability. This could relate to the physical dimension of NPs, causing them to
424 accumulate in the gut of the daphnids as result of their filter-feeding behavior. Accumulation in the
425 gut may cause CuONPs to dissolve more readily as result of the environment in the gut, as
426 suggested for AgNPs (Gaiser et al., 2011), but remaining CuO particles in the gut region of the
427 daphnids may also increase the internal exposure beyond the pulse duration. In line with this, higher
428 body burdens of copper have been measured in daphnids exposed to CuONPs, compared to CuCl₂
429 (Adam et al., 2015), although the effects on reproduction in that study were similar for CuCl₂ and
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 ≥ 60 live offspring
433 produced per surviving mother organism in unexposed control groups after 21 days (OECD, 2012).
434 This criterion was met for four of the twelve tests, short by a few in four tests (1 and 3 h CuCl₂, 1 h
435 and 3 h pulse AgNO₃) and short by more (a total of 37-49 offspring per daphnid) in the three tests
436 with AgNP pulses. The offspring production was however in the same range as for the pre-culture
437 and because there was a positive effect or no effect on number of offspring in the tests with lowest
438 control reproduction (AgNPs and AgNO₃ pulses), this lower production is not assumed to indicate
439 lower fitness of the organisms and thereby have influenced the test outcome.

440

441 Overall, the concentrations found to cause immobilization in *D. magna* of both silver forms
442 (AgNO_3 and AgNPs) and CuCl_2 after 2-3 h pulse exposures were similar to those found for 24 h
443 continuous exposure. For CuONPs the effects of pulses on *D. magna* mobility were slightly lower
444 than those observed after continuous exposure. In contrast, the reproductive effects were most
445 pronounced for CuONPs, whereas exposure to AgNPs in sub-lethal concentrations seemed to
446 slightly stimulate reproduction.

447

448 The 1, 2, and 3 h pulses of AgNO_3 and AgNPs caused acute effects at concentration levels
449 comparable to what has been reported in the literature for 48 h continuous exposure. Our results
450 showed that if a few hours of exposure to silver do not cause lethality in *D. magna*, then neither
451 delayed mortality nor effects on molting, body length and reproduction are likely to occur. While
452 the toxic mode of action governing acute toxicity of silver is well-known, the mechanism behind
453 reduced offspring production is less clear and may involve transfer of egg yolk protein or simply a
454 diversion of metabolic energy away from reproduction, towards metal detoxification (Wood et al.,
455 2002).

456 **4. Conclusion**

457

458 This study aimed to investigate the applicability of 1-3 h pulse exposure for stabilizing exposures
459 and disclosing acute and chronic effects of AgNPs, CuONPs, AgNO₃ and CuCl₂ in *D. magna*.

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

468

469 Pulse exposure tests with *D. magna* may serve for acute toxicity screening of engineered
470 nanoparticles as they represent environmentally relevant exposure scenarios and enable more stable
471 exposure scenarios to be maintained during the incubation in test medium, than standard continuous
472 exposure tests. Applicability was demonstrated in terms of acute immobility for AgNPs and
473 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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480 Council (grant no. 281579).

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613

614 **Tables**

615

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

Toxicity test	AgNO ₃	AgNPs	CuCl ₂	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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618

619 **Table 2.** EC₅₀-values with 95% confidence intervals in brackets for acute immobilization responses
 620 occurring after 24 and 48 hours standard continuous exposures, and after 0, 24 and 48 hours post-
 621 exposure following 1, 2 and 3 hour pulses to AgNPs, AgNO₃, CuONPs and CuCl₂.

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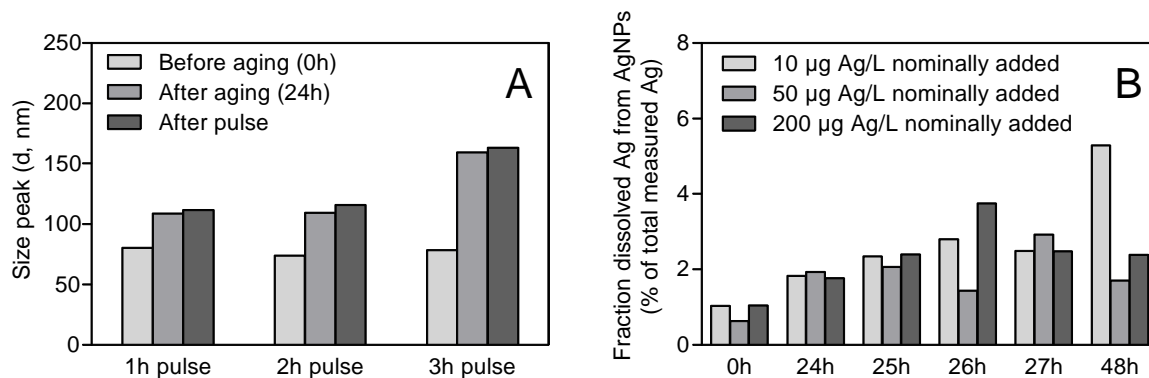
Exposure type	AgNPs	AgNO ₃	CuONPs	CuCl ₂
	µg Ag/L	µg Ag/L	mg Cu/L	mg Cu/L
Continuous				
EC _{50,24h}	130 [120 ; 150]	4.5 [4.0 ; 5.2]	6.5 [3.7 ; 46]	0.10 [0.080 ; 0.13]
EC _{50,48h}	n.d.	4.0 [n.e.]	5.2 [2.9 ; 25]	0.05* [n.e.]
1 h pulse				
EC _{50,0h post-exposure}	290 [200 ; 9700]	8* [n.e.]	130 [31;4.9×10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

623 n.d. Not determined.

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

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

626



628

629 **Figure 1.** Agglomeration and dissolution of AgNPs in modified M7. A) Mean size of the single size peak
 630 detected by DLS for AgNPs in modified M7 (of nominal concentration 200 µg Ag/L) immediately upon
 631 preparation (0 h), after aging (24 h), and after pulse exposure (1, 2 and 3 h, respectively). B) Dissolved silver
 632 measured in the supernatant of ultracentrifuged AgNP suspensions (of nominal concentrations 10, 50 and
 633 200 µg Ag/L) over time, as % of the total measured Ag concentrations at time 0, 24 and 48 h, respectively.

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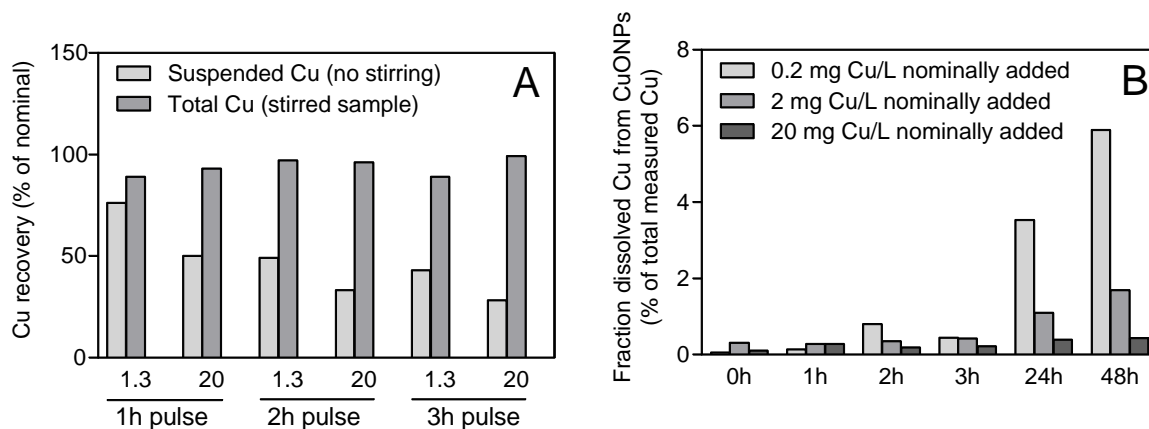
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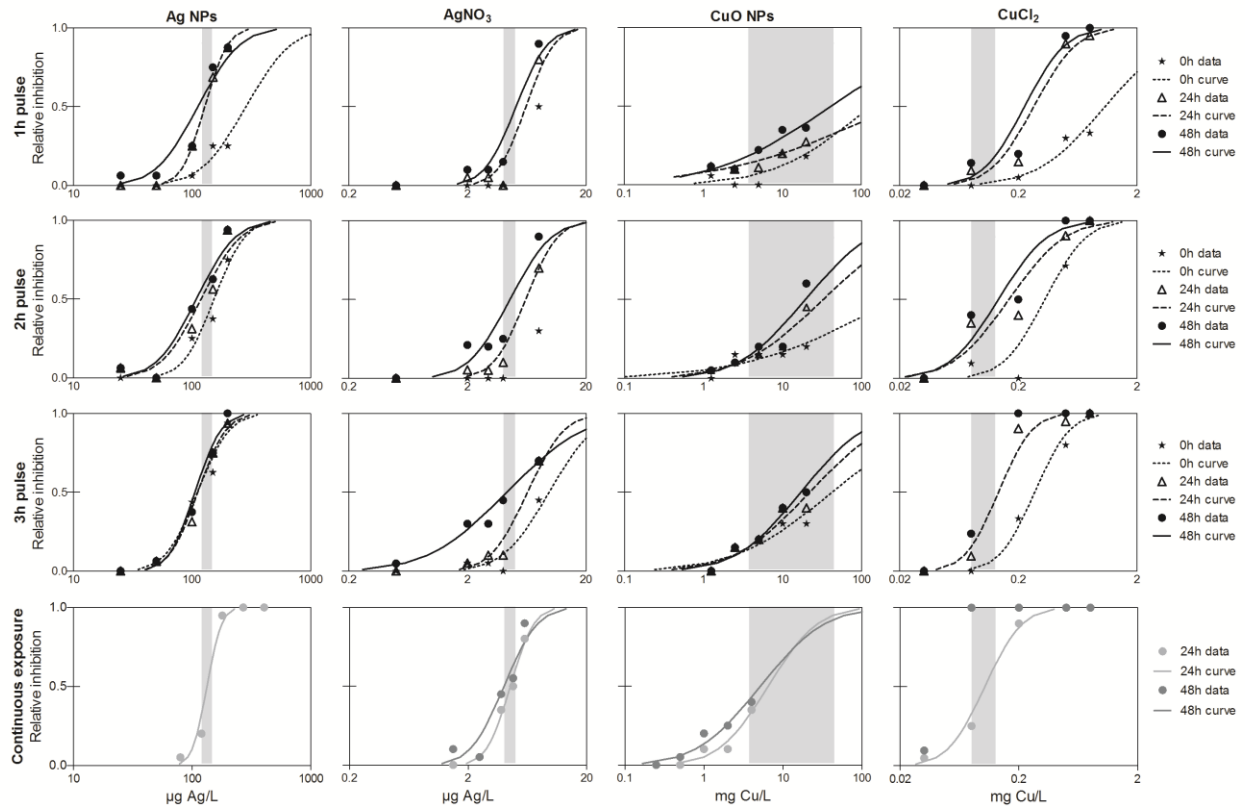
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645 **Figure 2.** Sedimentation and dissolution of CuONPs in modified M7. A) Measured copper concentration as
 646 percent of nominal in CuONP suspensions (1.3 and 20 mg Cu/L) after ended pulse exposures (1, 2 and 3 h)
 647 sampled first without stirring from center of suspensions, and then sampled during stirring, yielding the
 648 suspended and total copper, respectively. B) Fraction of dissolved copper measured in the supernatant of
 649 ultracentrifuged CuONP suspensions (of nominal concentrations 0.2, 2 and 20 mg Cu/L) determined 0, 1, 2,
 650 3, 24 and 48 hours after suspending the CuONPs (shown as percent of the total measured copper
 651 concentration at 0 h). All measured Cu concentrations are corrected for the Cu nutrient of the medium.

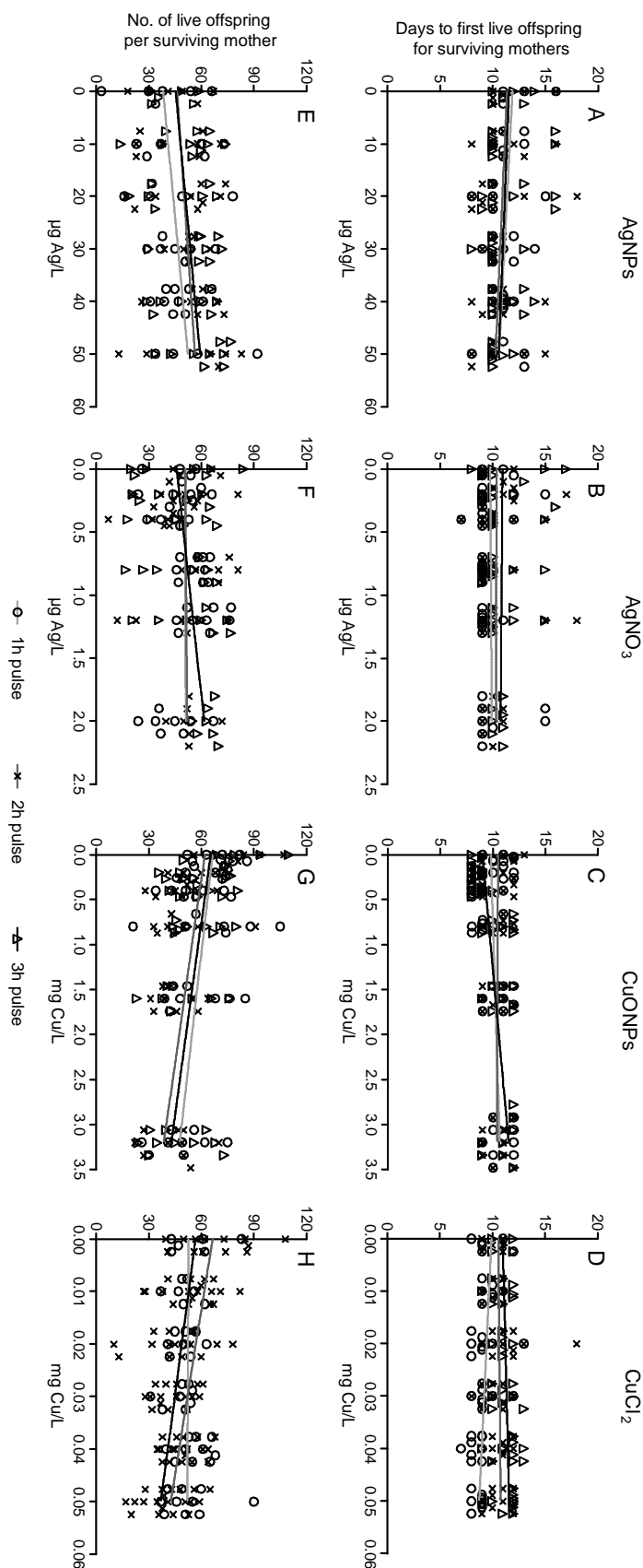
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Figure 3. Acute toxicity (immobilization) to *D. magna* of AgNPs, AgNO₃, CuONPs and CuCl₂ (columns 1-4) after 1, 2 and 3 hour pulse exposures (row 1-3) and 48 h standard continuous exposures (bottom row). Data are provided as number of immobile organisms relative to the total number of organisms for each concentration (relative inhibition) along with fitted concentration-response curves estimated with TOXCALC when possible from data. Immobility responses to pulses are shown for 0, 24 and 48 hour post-exposure observation periods. For standard tests, observations made after 24 and 48 hour continuous exposures are shown (for AgNPs only the 24 h data are available). The shaded areas depict the 95% confidence intervals of the EC_{50,24h} from continuous exposure tests (bottom row).



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665 **Figure 4.** Reproduction data for *D. magna* in the 21 days post-exposure observation period after 1, 2, and 3 h
 666 pulse exposure to AgNPs, AgNO₃, CuONPs and CuCl₂ (columns 1-4). Data are observations made over the
 667 21 days period for each surviving mother organism, along with linear regressions for 1, 2 and 3 h pulses,
 668 respectively. Row 1: Days to first live offspring. Row 2: Total number of live offspring produced in the post-
 669 exposure period per surviving mother organism. Parameters and statistics for linear regressions are provided
 670 in Table S2.