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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 suspensions. The resulting difficulties in controlling and characterizing exposure concentrations are detrimental to the generation of concentration-response data needed for hazard identification of NPs. This study explores the applicability of short-term (1, 2 and 3 h) pulse exposures as means to keep the exposure stable and at the same time disclose acute and chronic effects of AgNPs and CuONPs in *D. magna*. Dissolution, agglomeration and sedimentation were found to have less influence on exposure concentrations during 1-3 h pulses than for 24-48 h continuous exposures. For AgNPs, preparation of test suspensions in medium 24 h before toxicity testing (aging) increased stability during the short-term pulses. In pulse tests, organisms were exposed to the test materials AgNPs and CuONPs for 1, 2 and 3 h, and afterwards transferred to clean medium and observed for 48 h (post-exposure period) for acute effects and for 21 d for chronic effects. $AgNO_3$ and $CuCl_2$ were used as reference materials for dissolved silver and copper, respectively. For all test materials, a 3 h pulse caused comparable immobility in *D. magna* (observed after 48 h post-exposure) as 24 h continuous exposure, as evidenced by overlapping 95% confidence intervals of EC_{50}-values. In the 21 d post-exposure period, no trends in mortality or body length were identified. AgNPs and $AgNO_3$ pulses had no effect on the number of moltings, days to first live offspring or cumulated number of offspring, but the number of offspring increased for AgNPs (3 h pulse only). In contrast, CuONPs and $CuCl_2$ pulses decreased the number of moltings and offspring, and for CuONPs the time to first live offspring was prolonged. After CuONP exposures, the offspring production decreased more with increasing concentrations than for $CuCl_2$ exposures when taking the measured dissolved copper into account. This indicates a nanoparticle-specific effect for CuONPs, possible 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 more stable exposures and cause acute immobility of *D. magna* comparable to continuous 24 h exposures. Pulse exposure is likely relevant and applicable for other toxic and dissolving metal NPs, but this requires further research.

Keywords

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

1. Introduction

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

The freshwater crustacean *Daphnia magna* is commonly used for standardized toxicity testing of 24-48 h acute immobility and 21-days chronic effects regarding reproduction, growth and mortality (OECD, 2012, 2004). Daphnids have high ecological relevance and a relatively short life-cycle that 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 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; Zhao and Wang, 2011). One reason is likely that chronic tests are more labor and cost intensive,

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 *D. magna* are usually renewed every second to third day, to keep the exposure concentration stable, ensure sufficient amounts of nutrients and remove waste products. For ENPs, small changes to the stock preparation procedure may influence the prepared suspensions (Hartmann et al., 2015) and compromise the repeatability and reproducibility of the renewal steps. For the ENPs that undergo dissolution, such as AgNPs and CuONPs the issue of unstable exposure scenarios becomes further complicated by the dissolution kinetics. Also, the necessity of feeding during a chronic test becomes an influencing factor, as the feeding conditions have shown to influence the chronic effects of AgNPs to *D. magna* (Mackevica et al., 2015).

Short-term exposures in *D. magna* identified as "pulse" or "intermittent" exposures have been applied for toxicity testing of both organic and inorganic substances, with the effects being monitored immediately after or during a post-exposure period in clean media after the pulse (Andersen et al., 2006; Hoang and Klaine, 2008; Trac et al., 2015). Pulse toxicity tests have been applied mainly to simulate a more environmentally realistic and relevant exposure scenario, as many chemical pollutants are discharged into aquatic environments as "pulses" resulting from e.g. 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, agrochemicals with a short half-life in the environment have also been suggested to exhibit pulse-like exposures (Reinert et al., 2002). Analogously, ENPs are also expected to rapidly transform from their pristine form, due to for example dissolution, agglomeration, sedimentation, and coating 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 has found the fate of cerium oxide ENPs to differ for press and pulse exposures and the authors 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

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_2 (Seitz et al., 2013), ZnO (Adam et al., 2014; Zhao et al., 2012), and CuO (Adam et al., 2015; Rossetto et al., 2014; Zhao et al., 2012). These studies focus on a variety of influencing factors such as feeding conditions, exposure routes, exposure scenarios (semi-static vs. flow-through), mixture effects, ENP characteristics (e.g. size, agglomeration, coating), environmental factors (e.g. pH, media, dissolved organic matter) and effects of ENPs vs. released ions. However, to our knowledge, the influence of exposure duration, in terms of a short-term NP pulse, and the following acute and chronic effect to *D. magna* remains unexamined.

The aim of this study is to explore the applicability of a short-term pulse exposure to reveal acute and chronic effects of Ag and CuONPs to *D. magna*. We exposed neonate *D. magna* to 1, 2 and 3 h pulse exposures to AgNPs, CuONPs, silver nitrate ($AgNO_3$) and copper chloride ($CuCl_2$) and studied the acute (immobility) and chronic effects (mortality, body length, molting and reproduction) occurring within 48 h and 21 d post-exposure periods, respectively. The obtained responses were then compared to those of continuous exposures in acute and chronic tests with the same materials. Pulsed exposure presents an environmentally relevant exposure scenario and may also facilitate more stable exposure conditions for ENPs during testing. As the transformation processes of suspended ENPs are time-dependent, we expected less impact of such processes during 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 sedimentation of AgNPs and CuONPs was monitored over the course of the pulse (1-3 h) and continuous (48 h) exposures.

2. Materials and Methods

2.1 Media, chemicals and preparation of suspensions

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

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

2.2 Characterization of test suspensions

The total silver and copper concentrations were determined by ICP-MS (Agilent 7700, Agilent, Santa Clara, CA, USA), and for some silver samples ICP-OES (Varian Vista-MPX CCD simultaneous ICP-OES). Measurements were conducted for all test concentrations prior to toxicity testing and for the highest test concentrations after ended pulse exposures. Samples were added concentrated HNO_3 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.

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.

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.

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 µg 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_3 and the Ag and Cu concentration determined by ICP-MS.

Speciation of Ag and Cu from $AgNO_3$ and $CuCl_2$ in modified M7 was estimated by the chemical speciation modeling program Visual MINTEQ 3.0 (Gustafsson, 2010), assuming equilibrium with atmospheric CO_2.

2.3 Acute toxicity tests

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

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 guideline (OECD, 2012), however all organisms were kept in clean media throughout the observation period. Ten neonate daphnids (< 24 h old) was exposed in a 100 mL beaker containing 50 mL test suspension (or medium for the controls) in the dark at 20 ± 2 °C. The exposure concentration ranges (Table 1) were based on the outcome of the acute toxicity tests, to target a maximum 10% lethality.

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

2.5 Data treatment and statistical analyses

The measured Cu concentration in suspensions of CuONPs and $CuCl_2$ was 95 ± 10% of the nominal (average and standard deviation), thus nominal Cu concentrations were applied for data treatment. For $AgNO_3$ and AgNP suspensions, the average silver recovery was 69 ± 21% of the nominal concentrations. The lower $AgNO_3$ concentrations (0.2-8 µg 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,
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 TOXCALC^{TM} 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.

3. Results and discussion

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 concentrations in medium), no substantial loss of silver occurred over time during the 24 h aging of 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%. The samples for ICP-OES analysis were drawn from suspensions without stirring, thus data indicates no substantial sedimentation of AgNPs, and no visible sedimentation or other changes in the appearance of suspensions was observed over 48 hours. According to DLS measurements, the size distribution of AgNPs in medium (200 µg Ag/L) was monodisperse with a single size peak and a polydispersity index (PDI) in the range of 0.14-0.39 for all measurements. The size of AgNPs increased during the 24 h aging step prior to exposure, but then remained relatively stable during the following 1-3 hour pulses, see Figure 1A.

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 ≤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).

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
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

The chemical speciation was estimated by Visual MINTEQ for $AgNO_3$ and $CuCl_2$ 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 $AgNO_3$: $AgCl_{aq}$ (64%), $AgCl_2^-$ (27%), and Ag^+ (8%), and for $CuCl_2$: $CuCO_{3,aq}$ (65%), $CuEDTA^{2-}$ (13%), $CuOH^+$ (12%), and Cu^{2+} (4%).

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 more acutely toxic to *D. magna* than copper, see Figure 3. Mean effective concentrations (EC_{50}) and 95% confidence intervals for all the curves in Figure 4 are provided in Table 2. For all test materials, the effect level detected 48 h post-exposure from pulse exposures was comparable to the effect level upon 24 h continuous exposures, with EC_{50}-values having overlapping 95% confidence intervals. With few exceptions, the 24 and 48 h EC_{50}-values from 2 and 3 h pulses were not statistically significant different from those of 24 h continuous exposures, as evidenced by the overlapping 95% confidence intervals. Generally, fewer immobile organisms were observed immediately after the pulse exposures (0 h post-exposure) than after 24 and 48 hours post-exposure observation. This indicates that the organisms were indeed affected by the short-term pulses, 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 toxicokinetics and/or toxicodynamics than is the case for AgNPs. Ionic silver (Ag^+) is known to inhibit the active Na^+ uptake by blocking the N^+, K^+-ATPase, a process shown to occur within 2 hours and be complete within 12 h in *Daphnia magna* (Bianchini and Wood, 2003). The acute toxicity of copper may involve a similar physiological mechanism (Bianchini and Wood, 2003), but also the redox cycling of copper and following generation of reactive oxygen species has been 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 ≤ 20%. In three of the nine tests, a control mortality of 40-50%

occurred (control groups for 1 and 2 hour $AgNO_3$ pulse, and 3 hour CuONPs pulse, respectively). For the CuONP pulse, this was not reflected by an overall high level of mortality in the exposed groups ($\leq$ 20%), and the results of this test are therefore considered valid. For the $AgNO_3$ tests, high mortalities generally occurred across exposure groups, however the high mortalities in the control groups hamper the interpretation of these mortality results.

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 ($p > 0.05$).

The number of moltings per surviving mother organism was not affected by the pulse-duration, however the linear regressions on pooled data indicate a slight decrease in the number of moltings with concentration for $CuCl_2$ and CuONPs, but not for $AgNO_3$ and AgNPs (Figure S2).

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

For AgNPs and $AgNO_3$, the time to first live offspring did not change with silver concentration or 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 pulse exposure to AgNPs ($p < 0.05$). A stimulating effect on the number of offspring has previously been reported for the same type of AgNPs at 10 µg Ag/L in a static renewal setup (Mackevica et al., 2015) and for other citrate-coated AgNPs at 2 µg Ag/L (Pokhrel and Dubey, 2012). This hormetic effect of silver could result from the antibacterial effects of silver causing better conditions for the test organism as also suggested by Mackevica et al. (2015). Other studies have found detrimental effects of AgNPs on the survival, growth and/or reproduction of *D. magna* at 10-50 µg Ag/L

389 (Mackevica et al., 2015; Zhao and Wang, 2011). For $AgNO_3$, 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 $AgNO_3$ 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 $CuCl_2$ 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 $CuCl_2$ 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 ($CuCl_2$)
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 $CuCl_2$, 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.

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 dissolved copper concentrations based on the dissolution measurements, and compared with the data for $CuCl_2$ in Figure 4H. Since the linear regressions of the number of offspring as a function of the dissolved copper concentrations for 1, 2 and 3 h CuONPs pulses were not statistically significant different with respect to slope and constant term, these data were pooled to simplify the comparison. For $CuCl_2$, pooling of data from 1, 2 and 3 h pulses was not possible due to different slopes and/or elevations. Instead, the data with the steepest slope was used for the comparison (2 h pulse). Comparing the two datasets and the linear regressions (Figure S3), the effects of dissolved Cu from CuONPs were more pronounced than for $CuCl_2$, indicating a nano-specific effect on toxicity or bioavailability. This could relate to the physical dimension of NPs, causing them to accumulate in the gut of the daphnids as result of their filter-feeding behavior. Accumulation in the gut may cause CuONPs to dissolve more readily as result of the environment in the gut, as suggested for AgNPs (Gaiser et al., 2011), but remaining CuO particles in the gut region of the daphnids may also increase the internal exposure beyond the pulse duration. In line with this, higher body burdens of copper have been measured in daphnids exposed to CuONPs, compared to $CuCl_2$ (Adam et al., 2015), although the effects on reproduction in that study were similar for $CuCl_2$ and CuONPs when the results were expressed as dissolved copper.

The OECD guideline no. 211 includes a validity criteria of a mean number of ≥ 60 live offspring produced per surviving mother organism in unexposed control groups after 21 days (OECD, 2012). This criterion was met for four of the twelve tests, short by a few in four tests (1 and 3 h $CuCl_2$, 1 h and 3 h pulse $AgNO_3$) and short by more (a total of 37-49 offspring per daphnid) in the three tests with AgNP pulses. The offspring production was however in the same range as for the pre-culture and because there was a positive effect or no effect on number of offspring in the tests with lowest control reproduction (AgNPs and $AgNO_3$ pulses), this lower production is not assumed to indicate lower fitness of the organisms and thereby have influenced the test outcome.

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).

4. Conclusion

This study aimed to investigate the applicability of 1-3 h pulse exposure for stabilizing exposures and disclosing acute and chronic effects of AgNPs, CuONPs, $AgNO_3$ and $CuCl_2$ in *D. magna*. Overall, the impact of dissolution, agglomeration and sedimentation on the stability of ENPs in daphnia test medium was less during 1-3 h, compared to the 24-48 h representative of a standard acute immobility test. The level of immobility observed for *D. magna* 48 h after a short-term pulse exposure (1, 2, and 3 h) to the test materials was generally comparable to the effect levels observed after 24 h continuous exposure. For CuONPs the effects of pulse exposures were slightly lower than for continuous exposure, although not statistically. With regards to chronic effects, the 1-3 h pulses impacted mortality, body length and reproduction less than what is reported in literature for chronic tests with continuous exposures to the same test materials.

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 very toxic and partly dissolving metal NPs.

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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

617

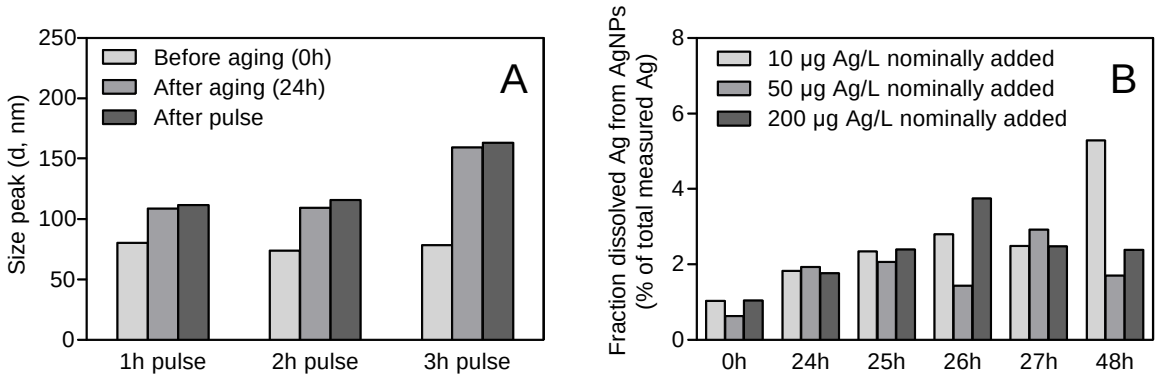
618

619 **Table 2.** EC_{50}-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, $AgNO_3$, CuONPs and $CuCl_2$.
622

Exposure type	**AgNPs** µg Ag/L	**$AgNO_3$** µg Ag/L	**CuONPs** mg Cu/L	**$CuCl_2$** 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				
$EC_{50,0h\ post\text{-}exposure}$	290 [200 ; 9700]	8* [n.e.]	130 [31;4.9×10^{8}]	1.1 [0.70 ; 4.3]
$EC_{50,24h\ post\text{-}exposure}$	130 [110 ; 150]	6.2 [n.e.]	280 [n.e.]	0.27 [0.21 ; 0.34]
$EC_{50,48h\ post\text{-}exposure}$	110 [88 ; 140]	5.1 [4.3 ; 6.5]	42 [15 ; 89000]	0.22 [0.036 ; 0.75]
2 h pulse				
$EC_{50,0h\ post\text{-}exposure}$	150 [130 ; 200]	> 8* [n.e.]	260 [n.e.]	0.34 [n.e.]
$EC_{50,24h\ post\text{-}exposure}$	120 [n.e.]	6.2 [5.2 ; 8.2]	33 [15 ; 520]	0.17 [0.12 ; 0.22]
$EC_{50,48h\ post\text{-}exposure}$	110 [n.e.]	4.6 [3.7 ; 6.0]	19 [12 ; 66]	0.14 [0.031 ; 0.42]
3 h pulse				
$EC_{50,0h\ post\text{-}exposure}$	110 [90 ; 130]	9.8 [7.0 ; 27]	43 [17 ; 4800]	0.27 [0.22 ; 0.34]
$EC_{50,24h\ post\text{-}exposure}$	110 [92 ; 130]	6.4 [5.2 ; 9.1]	22 [12 ; 110]	0.14 [n.e.]
$EC_{50,48h\ post\text{-}exposure}$	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

627 **Figures**



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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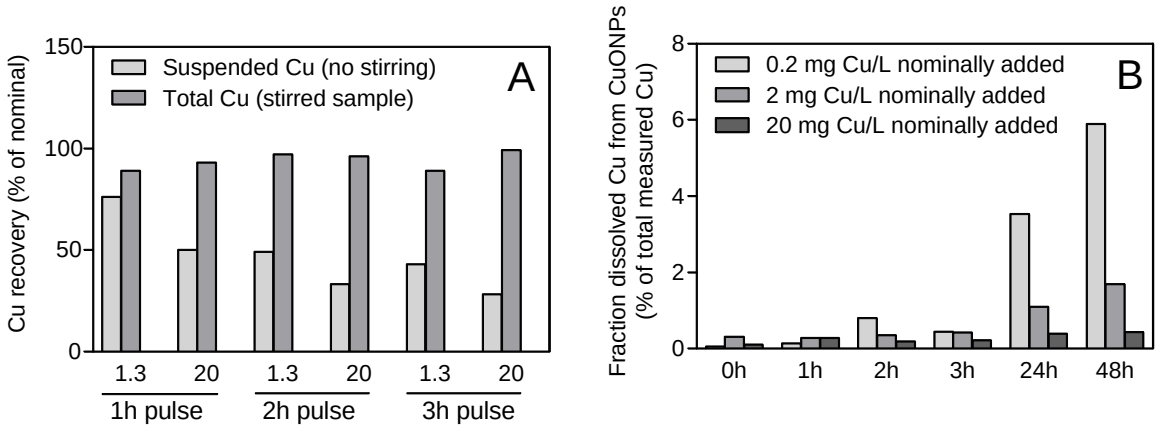

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645 **Figure 2.** Sedimentation and dissolution of CuONPs in modified M7. A) Measured copper concentration as
646 percent of nominal in CuONP suspensions (1.3 and 20 mg Cu/L) after ended pulse exposures (1, 2 and 3 h)
647 sampled first without stirring 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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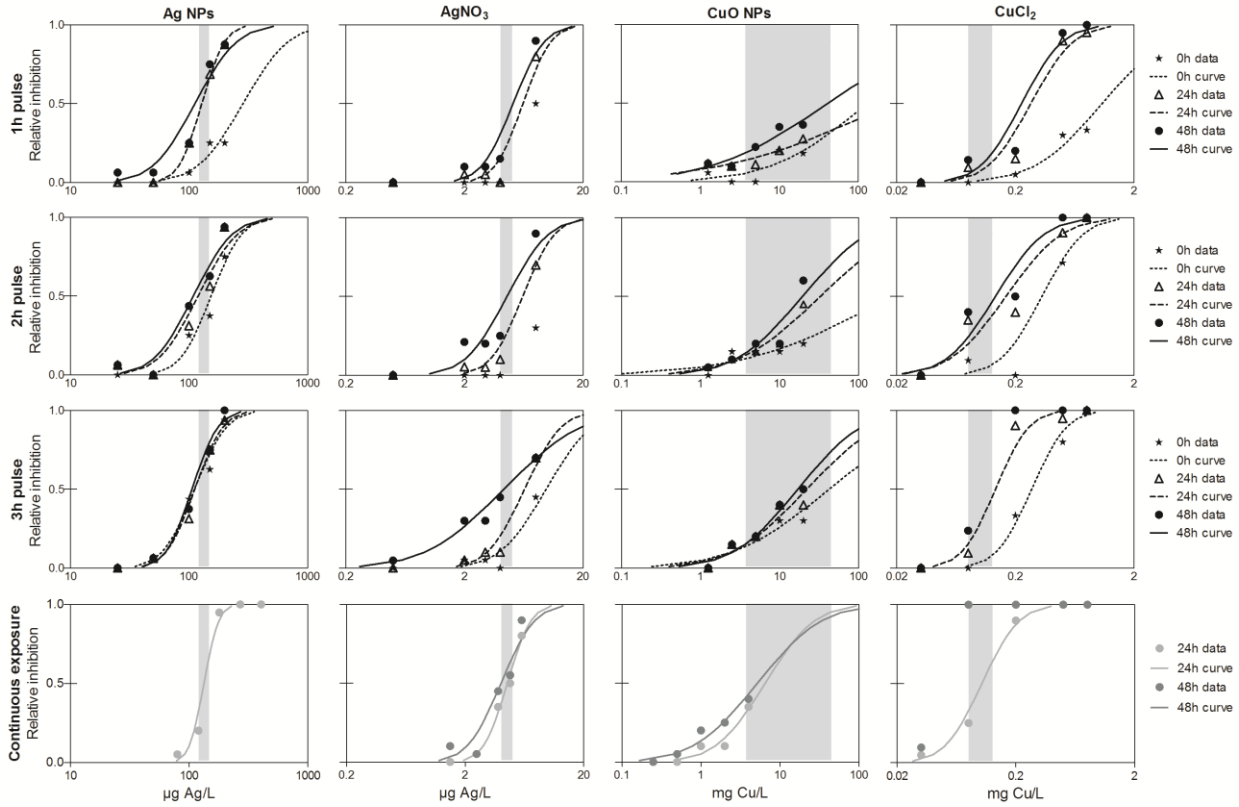

Figure 3. Acute toxicity (immobilization) to *D. magna* of AgNPs, $AgNO_3$, CuONPs and $CuCl_2$ (columns 1-4) after 1, 2 and 3 hour pulse exposures (row 1-3) and 48 h standard continuous exposures (bottom row) . Data are provided as number of immobile organisms relative to the total number of organisms 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).

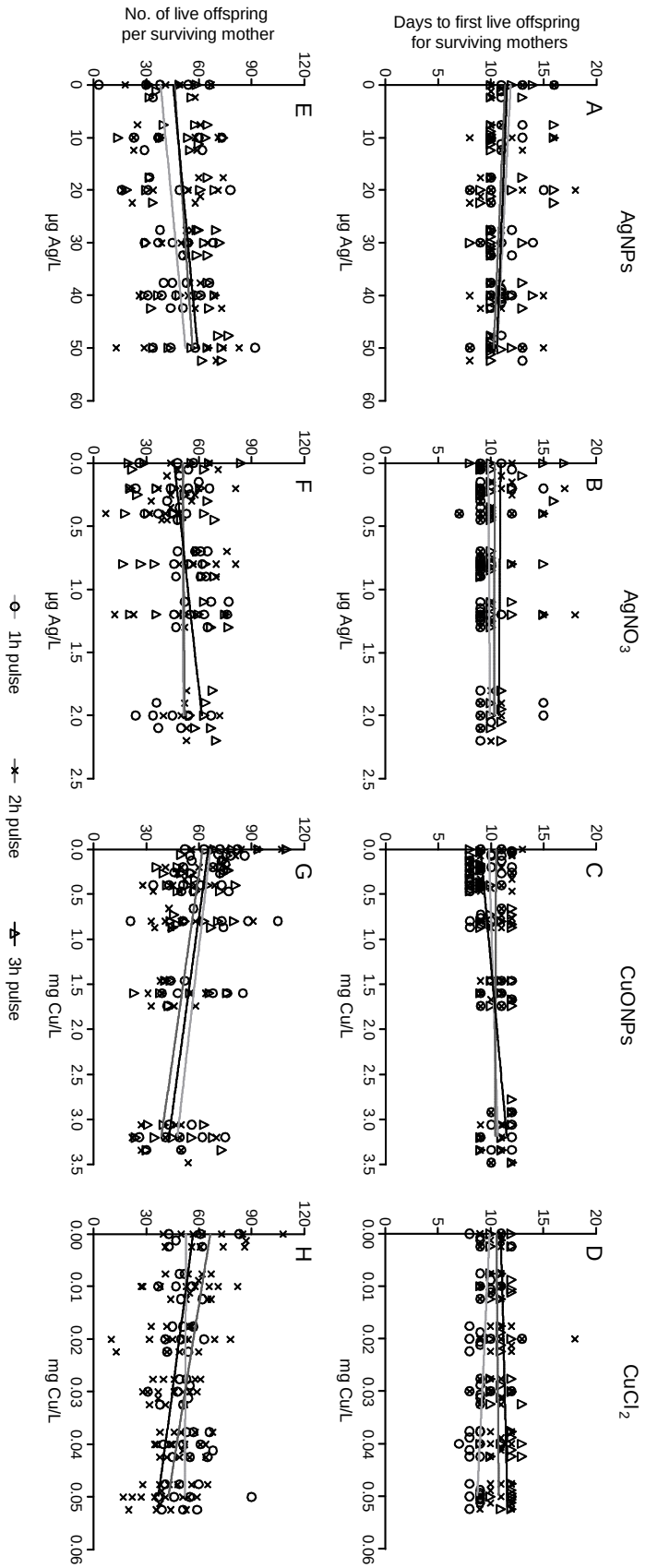

Figure 4. Reproduction data for *D. magna* in the 21 days post-exposure observation period after 1, 2, and 3 h pulse exposure to AgNPs, $AgNO_3$, CuONPs and $CuCl_2$ (columns 1-4). Data are observations made over the 21 days period for each surviving mother organism, along with linear regressions for 1, 2 and 3 h pulses, respectively. Row 1: Days to first live offspring. Row 2: Total number of live offspring produced in the post-exposure period per surviving mother organism. Parameters and statistics for linear regressions are provided in Table S2.