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Effects of metal nanoparticles on freshwater rotifers may persist across generations

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ABSTRACT

Nanotechnology has become one of the fastest growing industries in the current century because nanomaterials (NMs) are present in an ever-expanding range of consumer products increasing the chance of their release into natural environments. In this study, the impacts of two metal nanoparticles (Ag-NPs and CuO-NPs) and their equivalent ionic forms (Ag^{+} and Cu^{2+}) were assessed on the lentic freshwater rotifer *Brachionus calyciflorus* and on its ability to adapt and recover through generations. In our study, Ag-NPs and CuO-NPs inhibited the rotifer population growth rate and caused mortality at low concentrations (< 100 μg L^{-1}). Ag-NPs and CuO-NPs decreased in the medium when organisms were present (48 h exposure: 51.1 % and 66.9 %, respectively), similarly Ag^{+} and Cu^{2+} also decreased from medium in presence of the organisms (48 h: 35.2 % and 47.3 %, respectively); although the metal concentrations removed from the medium were higher for nanoparticles than metal ions, metal ions showed higher effects then their respective nanoparticle forms. Rotifer populations exposed for 4 generations to the toxicants were able to recover the population growth rate, but some rotifers showed developmental delay and inability to reproduce even after the removal of the toxicants. Intracellular accumulation of reactive oxygen species as well as plasma membrane damage were found in the rotifers at concentrations corresponding to EC_{10} (Ag-NPs = 1.7 μg L^{-1}, Ag^{+} = 4.5 μg L^{-1}, CuO-NPs = 46.9 μg L^{-1}, Cu^{2+} = 35 μg L^{-1}) of the population growth rate. Our results showed, for the first time, that effects of metal nanoparticles and metal ions on rotifer populations may persist along several generations. This should be taken into account when assessing risks of metal nanoparticles in freshwaters.

1. Introduction

Nanotechnology is one of the rapidly growing industries of the 21st century (Bondarenko et al., 2013), expanding its influence on several fields, including medicine (Barnett et al., 2007), cosmetics (Lens, 2009), renewable energies (Wei et al., 2008), environmental remediation (Tungittiplakorn et al., 2004) and electronic devices (Kachynski et al., 2008). The increasing production, consumption and release of nanomaterials with unique physico-chemical properties (Moore, 2006) into the aquatic environment have raised concern on their behaviour and toxicity, posing a new challenge to the environment and human health (Darlington et al., 2009).

Ag-NPs and CuO-NPs are among the most commercially used metal nanoparticles, encompassing a large variety of applications in various fields. Ag-NPs are mostly used in inks, textiles, soaps, microelectronics and medical imaging (Frattini et al., 2005; Wu et al., 2006; Jain et al., 2008; Perelaer et al., 2009). The growing market of Ag-NPs is mainly associated with the antimicrobial activity of silver (Luoma, 2008) and with its low cost of production (Capek, 2004). On the other hand, CuO-NPs are used, for example, in semiconductors, electronic chips, solar cells, lithium batteries, gas sensors and as antimicrobial agents (Ren et al., 2009; Bondarenko et al., 2013).

Data on concentrations of Ag-NPs and CuO-NPs in aquatic environments are scarce. Predicted environmental concentrations of Ag-NPs range from 0.0028 to 0.619 μg L^{-1} (Musee, 2011) in surface waters, but much lower concentrations (0.3–6.6 ng L^{-1}) were reported in the Meuse River from the Netherlands (Peters et al., 2018). Few information exists on CuO-NPs environmental concentrations, but copper concentration in industrial wastewaters can exceed 100 mg L^{-1} with almost half of the concentration corresponding to CuO-NPs (Huang et al.,

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2006).

Some studies have shown negative effects of Ag-NPs and CuO-NPs on freshwater organisms from producers (e.g. *Raphidocelis subcapitata*), primary consumers (e.g. *Daphnia magna*) to secondary consumers (e.g. *Pimephales promelas*) (Garner et al., 2015), with evidence that at least part of the nanoparticle toxicity is due to oxidative stress (Pradhan et al., 2015). Despite these reports, the potential impacts of metal NPs in aquatic systems are still uncertain. The European Chemicals Agency, US Environmental Protection Agency and World Health Organization are trying to establish directives and legal frameworks to protect the quality of freshwaters from these emergent chemical contaminants (Esplugas et al., 2007).

Freshwater rotifers are relatively small (< 200 μm) metazoan invertebrates that are widely distributed and are ecologically relevant because to their role in secondary production in lentic ecosystems (Wallace, 2002; Dahms et al., 2011). The ease of cultivation and laboratory maintenance, small size, preferably parthenogenic reproduction, high population density, short generation time, and sensitivity to a vast range of toxicants (Hagiwara et al., 1997; Gómez et al., 2002; Hagiwara et al., 2007) increase the potential of rotifers as model organisms for ecotoxicological tests.

Standard laboratory bioassays with rotifers include acute (ASTM, 2002) and chronic tests (Snell and Janssen, 1995) that mainly focuses on assessing mortality and reproduction (population growth rate). At cellular level, endpoints such as oxidative stress have shown to be a suitable biomarker in the rotifer model (Dahms and Hellio, 2009). The monogonont rotifer *B. calyciflorus* has been used for ecotoxicity tests for a large variety of xenobiotics, including metals and nanoparticles, and has proven suitable to allow quick data collection at lethal and sub-lethal level (Marcial et al., 2005; Snell and Hicks, 2011).

Metals and emergent contaminants have been reported to induce negative transgenerational effects on terrestrial and freshwater invertebrates (Yu et al., 2013; Castro et al., 2018) most of which are likely to be the consequences of epigenetic changes (Vandegehuchte et al., 2010; Baker et al., 2014). Castro et al. (2018) showed adverse effects in the fitness of the F1 generation of *Daphnia magna* after maternal exposure to paracetamol and copper sulphate. Also, microplastics had multigenerational effects causing the extinction of *D. magna* populations in 2 exposed generations, but when F1 was released from contaminants, a recovery was observed up to the F3 generation (Martins and Guilhermino, 2018). The knowledge on the transgenerational effects of NPs to freshwater invertebrates is limited (Yu et al., 2013; Schultz et al., 2016; Rossbach et al., 2019; Fan et al., 2019), however, this information can be relevant since metal NPs can persist in the environment and can exert effects throughout generations.

This study aims to evaluate the lethal and sub-lethal effects of two metal nanoparticles (Ag-NPs and CuO-NPs) on rotifer populations by assessing if and how i) environmentally relevant concentrations can affect the fitness and population size, and ii) future generations can be affected by the parental exposure. We hypothesised that i) NPs would exhibit lethal and sub-lethal effects towards rotifer populations, ii) toxicity could be related to oxidative stress and plasma membrane damage, even at very low exposure concentrations, and iii) effects would increase along generations if continuously exposed, but would be attenuated along generations when offsprings are released from NP exposure.

2. Materials and methods

2.1. Culture and maintenance of rotifers

Rotifer populations were fed on *Raphidocelis subcapitata* cultures, which were maintained in 2 L flasks in COMBO algae modified medium (Kilham et al., 1998; Table S1), with constant aeration (filtered through a syringe filter with 0.2 μm) at 20 ± 1 °C and continuous light. The medium and materials were sterilised in an autoclave (30 min at 120 °C and 5 bar).

B. calyciflorus was obtained from MicroBioTests Inc. as dormant cysts and were stored at 4 °C in darkness and hatched on demand. A standard freshwater medium (96 mg L^{-1} of $NaHCO_3$, 60 mg L^{-1} of $CaSO_4.2H_2O$, 60 mg L^{-1}, 60 mg L^{-1} of $MgSO_4.7H_2O$ and 4 mg L^{-1} of KCl; Table S2) with moderately hard water (U.S. Environmental Protection Agency, 1985) was prepared to hatch the cysts. Values of ionic strength in the standard freshwater medium did not vary with time and with the addition of contaminants (pH = 7.5 ± 0.3, conductivity = 250 ± 0.5 μS/cm). Hatching was done in 10 mL of standard freshwater medium in a climate chamber for 16–18 h at 25 °C with continuous illumination. Before assays, freshly hatched rotifers were fed with Roti-Rich™ suspension.

2.2. Stock suspensions and characterization of metal nanoparticles

Aqueous suspension of citrate-coated Ag-NPs (1 g L^{-1}; 20–30 nm size) was purchased from NanoSys GmbH (Wolfhalden, Switzerland). A stock suspension of 20 mg L^{-1} was prepared in standard freshwater medium, vortexed for 2 min, to disperse the nanoparticles, and stored in the absence of light until use. The stock suspension was diluted in the standard freshwater medium to prepare each nominal concentration. Stock suspension (80 mg L^{-1}) of CuO-NPs (< 50 nm powder of 99.5 % spherical CuO-NPs; Sigma-Aldrich) was prepared in standard freshwater medium, vortexed and stored in the dark until use.

Silver nitrate (BioXtra, > 99 %, titration) ($AgNO_3$, CAS Number: 7761−88-8) and copper chloride dihydrate (ACS reagent, ≥ 99 %) ($CuCl_2.2H_2O$, CAS Number: 10125−13-0) were purchased from Sigma-Aldrich. Stock solutions of Ag^+ and Cu^{2+} were prepared as those of Ag-NPs and CuO-NPs.

The hydrodynamic size distribution (HDD), zeta potential, and polydispersity index (PDI) of Ag-NPs and CuO-NPs in suspensions at the highest concentrations (500 μg L^{-1} of Ag and 2000 μg L^{-1} of Cu respectively) were monitored by dynamic light scattering (Malvem Zetasizer Nano ZS; Malvern Instruments). HDD samples were prepared as follows: i) T0 (beginning of the exposure) and T48 (end of the exposure) without rotifers or algae, and ii) T0 and T48 with rotifers and algae. In well plates, 1 rotifer per mL and 2×10^6 cells per mL of algae food (*Raphidocelis subcapitata*) were added to each treatment (3 replicates). Samples for DLS were collected at each time (T0 and T48) and filtered through 0.22 μm syringe filter to remove rotifers and algae in does experimental conditions.

Samples for metal quantification in nanoparticles and ionic forms were prepared as for DLS measurements. Samples were acidified (HNO_3, 5% v/v) prior to metal quantification by inductively coupled plasma mass spectrometry (ICP-MS) at Scientific and Technological Research Assistance Centre, University of Vigo, Spain. Figures of merit can be found in Table S8.

Metal quantification was assessed as above under the following conditions: i) T0 (beginning of the exposure) and T48 (end of the exposure) without rotifers or algae, and ii) T0 and T48 with rotifer and algae (3 replicates).

Parallel to metal quantification, samples for assessing metal dissolution from nanoparticles were prepared as above under the following conditions: T0 without organisms, T0 with organisms, T48 without organisms and T48 with organisms (3 replicates). Samples were loaded in Amicon Ultra-15 centrifugal filter units (3 kDa m.w. cut-off: < 2 nm of estimated pore size; Merck Millipore, Germany) and centrifuged at 7500 *g*. 30 min: 3 times) to quantify dissolved metals by ICP-MS. Maximum nominal concentrations of Ag-NPs as total Ag: 500 μg L^{-1}; Ag^+: 500 μg L^{-1}; CuO-NPs as total Cu: 2000 μg L^{-1}; Cu^{2+}: 2000 μg L^{-1} were chosen to guarantee inhibition of population growth rate at 100 %.

2.3. Mortality and reproduction assays

For 24 h rotifer mortality test, we assessed the toxicity of Ag-NPs,

Ag^+, CuO-NPs and Cu^{2+} to *B. calyciflorus* (ISO, 2001) by placing 5 juveniles in each well in 24 multi-well test plates. We used 8 concentrations of each contaminant (Ag-NPs as total Ag: 0.8–500 μg L^{-1}; Ag^+: 0.8–500 μg L^{-1}; CuO-NPs as total Cu: 39.1–5000 μg L^{-1}; Cu^{2+}: 2.3–40 μg L^{-1}) and a negative control with 4 replicates each, in a test volume of 1 mL per well. No feeding was done prior or during the 24 h test.

At 48 h sub-lethal test, we assessed the toxicity of Ag-NPs, CuO-NPs, Ag^+ and Cu^{2+} to reproduction and population growth of *B. calyciflorus* (ISO, 2001). After hatching, 1 juvenile of *B. calyciflorus* was placed each well in 24 multi-well test plates. We used 8 concentrations of the contaminants (Ag-NPs as total Ag: 1.56–200 μg L^{-1}; Ag^+: 1.56–200 μg L^{-1}; CuO-NPs as total Cu: 15.62–2000 μg L^{-1}; Cu^{2+}: 1.56–200 μg L^{-1}) and controls with 8 replicates each in a volume of 1 mL per well. Rotifers were feed 2 h prior to its introduction into the wells with Roti-Rich™ pre-feeding suspension and during the experimental period with a fresh suspension of the green algae *R. subcapitata* (2×10^6 cells per mL). Both assays were performed at 25 °C in the absence of light. At the end of the experiments, the total number of living and dead organisms per well was counted under a dissection microscope (Leica S8APO).

Population growth rate (r) was calculated as:

$$r = (\ln N_{final} - \ln N_{start}) / T$$

Where:

N_{final} = mean number of rotifers after 2 d incubation

N_{start} = mean number of rotifers at T0 (= 1)

T = time of exposure in days (= 2)

The validation of the test was achieved when the mean population growth rate of controls > 0.55 d^{-1}, the average percentage of effect at the lowest concentration < 50 % and reproduction occurred in at least 7 of the 8 replicates in the control.

2.4. Reactive oxygen species assessment

Ten juveniles were placed in each well in 6 multi-well plates, in a volume of 5 mL of standard freshwater medium per well (Table S1). Modelled concentrations as EC_{10} (effect concentration reducing 10 % of population growth rate) were tested against a negative control with clean freshwater medium (6 replicates) for a period of 48 h. After this period rotifers were frozen with liquid nitrogen and stored in -80 °C.

The rotifers from this 48 h sub-lethal test were prepared with the following fluorescence markers: 5 μM CM-H_2DCFDA, 15 μM propidium iodide and 100 μL of the anti-fading reagent containing 4′,6-diamidino-2-phenylindole (DAPI). Rotifers were unfrozen, centrifuged (10,000 *g* for 5 min) to remove the excess of medium. Then, rotifers were suspended in freshwater medium and incubated with CM-H_2DCFDA and propidium iodide for 15 min in the dark. After this period, the dye was removed by centrifugation (10,000 *g*; 5 min), and clean freshwater medium was added to resuspend rotifers. Finally, the anti-fading reagent was added before loading the samples onto the microscope slides. Each slide was scanned through an epifluorescence microscopy (160× and 200×, Leica DM5000B), and images were acquired with a digital camera (Leica DFC 350 FX R2) using the software LAS AF (Ver. 1.4.1).

2.5. Transgenerational assay

An adapted version of the 48 h rotifer reproduction test was performed to assess the toxicity of Ag-NPs, Ag^+, CuO-NPs and Cu^{2+} on the population growth and maturity state of *B. calyciflorus* through the generations over a period of 10 days. For all tested NPs and ions, the modelled EC_{50} was used as the exposure concentration. At the start of the experiment, F0 generation was exposed to EC_{50} concentration of each contaminant (Ag-NPs, Ag^+, CuO-NPs and Cu^{2+}) for 48 h. At 48 h, freshly born rotifers (less than 24 h old) were transferred to new wells to start the exposure of the F1 generation. From each replicate, one rotifer was exposed to contaminants (Ag-NPs, Ag^+, CuO-NPs and Cu^{2+}) and another rotifer was released from contaminants by transferring it to clean medium. At the end of the new 48 h (F1 generation), one rotifer with less than 24 h was transferred to a new contaminant treatment in order to start the F2 generation (Fig. S1). This procedure was repeated until reaching the F4 generation. At the end of each 48 h, live and dead rotifers were recorded to estimate population growth rate, and rotifers with more than 24 h were sampled and frozen for assessing the intracellular ROS accumulation and plasma membrane damage as described above (see 2.4).

2.6. Data analyses

Significant differences in metal concentrations at the beginning (T0) and at the end of the experiment (T48) were determined by a *t*-test. Significant differences in metal removal from the medium by organisms and in metal dissolution from nanoparticles were determined by two-away ANOVAs (Table S6 and Table S7) followed by Tukey's multiple comparison post-hoc tests. To test if the hydrodynamic size, zeta potential or PdI varied with the exposure time and the presence of rotifers and algae, two-way ANOVAs were used (Table S3), followed by Tukey's multiple comparison post-hoc tests.

Effect concentrations (EC_{10}, EC_{20} and EC_{50}) with respective 95 % confidence limits were calculated by Probit regression (Finney, 1971) for mortality data (using SPSS statistics 17.0) and by logistic regression for population growth rate (using STATISTICA 8.0 software). The determination of NOEC (no-observed-effect-concentration) and LOEC (low-observed-effect-concentration) of each contaminant for mortality and population growth rate were estimated by one-way ANOVA (Table S4), followed by the Dunnett's test to identify treatments that differed significantly from the control.

For each generation in the transgenerational test, the effects of each contaminant on exposed or pre-exposed rotifers were assessed by one-way ANOVAs (Table S5) followed by the Dunnett's tests. All ANOVAs and *t*-test analysis were performed in GraphPad Prism 7.

3. Results

3.1. Characterization of nanoparticles and quantification of metal concentrations

In the absence of rotifers and algae, no change in metal concentration in the medium was observed along time (*t*-test $P > 0.05$). Metal concentration in the medium at T0 in the absence of rotifers and algae was used as the initial concentration, to calculate the percentage of metal in the medium (Fig. S2). At T0, the presence of rotifers and algae led to a significant decrease ($P < 0.05$) in metal concentration for all metal forms, and this decrease was higher for nanoparticles than for their ionic forms ($P < 0.05$; Fig. S2). Rotifers and algae accumulated and/or removed a significant amount of CuO-NPs from the medium with time (T0 = 48.7 ± 2.8 % versus T48 = 66.9 ± 1.5 % $P > 0.05$; Fig. S2). Rotifers and algae also reduced significantly Cu^{2+} concentration in the medium with time (Cu^{2+} removal T0 = 23.1 ± 1% to T48 = 47.3 ± 1.4 %, $P < 0.05$; Fig. S2). On the other hand, concentration of Ag-NPs and Ag^+ in the medium decreased with time in the presence of rotifers and algae (Ag-NP T0 = 64.6 ± 2.3 % to T48 = 51.1 ± 1.7 %; Ag^+ T0 = 46.2 ± 2.1 % to T48 = 35.2 ± 0.7 %, *t*-test; $P < 0.05$; Fig. S2).

The presence of rotifers and algae had significant effects on average HDD of Ag-NPs (two-way ANOVA, $P < 0.05$), while exposure time showed no significant effect ($P > 0.05$). Significant decrease in the HDD of Ag-NPs was observed in the presence of rotifers and algae at the beginning (T0: from 152.8 nm to 113.7 nm, $P < 0.05$; Fig. 1) and at the end of exposure (T48: from 143.4–124.1 nm, $P < 0.05$; Fig. 1). Zeta potential of Ag-NPs was affected by time and by the presence of organisms (two-away ANOVA, $P < 0.05$; Fig. 1). At T0, the presence of

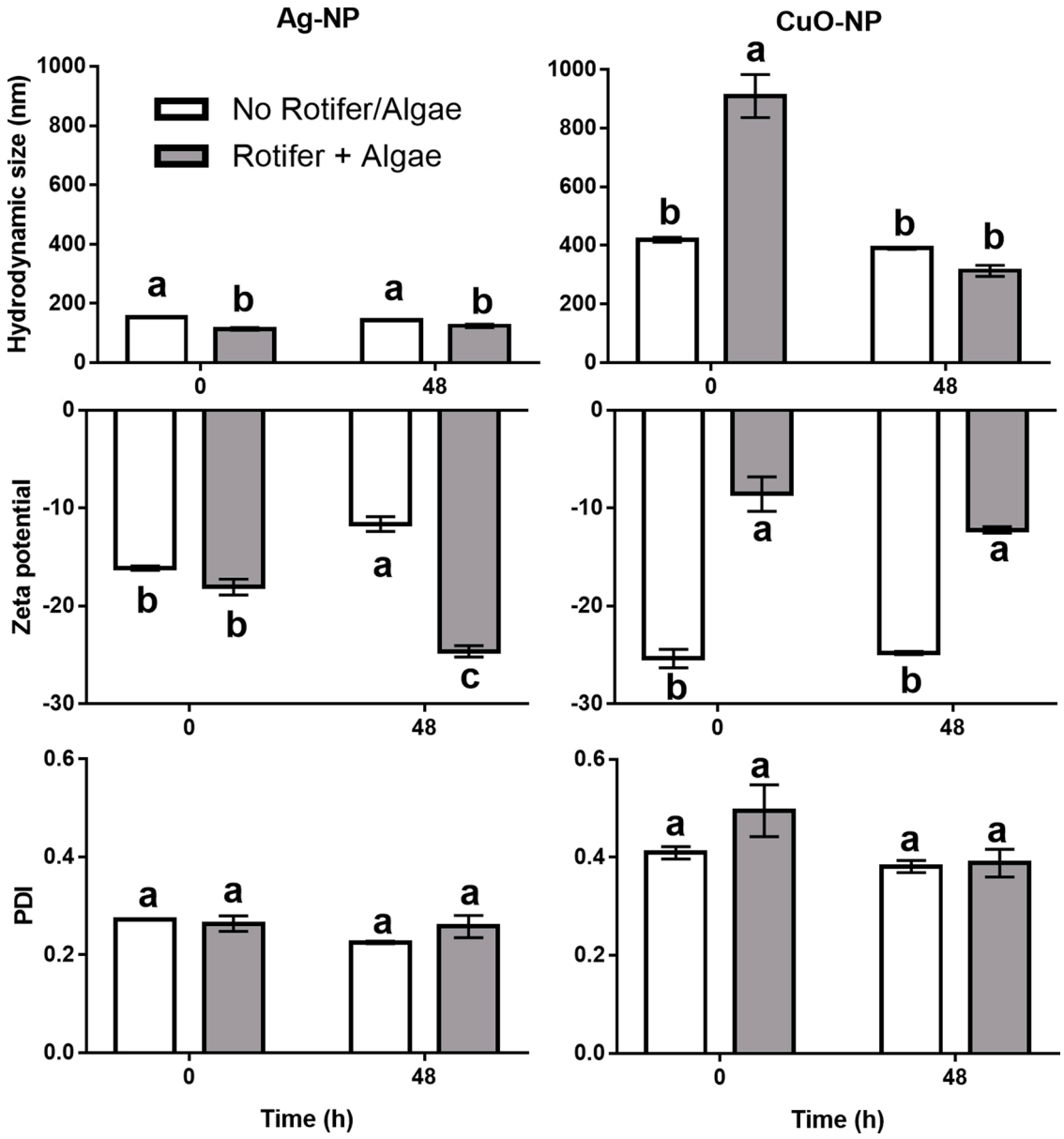

Fig. 1. Hydrodynamic diameter (nm), zeta potential and polydispersity index of Ag-NPs, and CuO-NPs at T0 and T48 in the presence and absence of rotifers and algae. Values are mean ± Standard error, n = 3 significantly different treatments are represented by different lowercase lettering (two-way ANOVA, Tukey's test $P < 0.05$).

organisms did not change the zeta potential of Ag-NPs ($P > 0.05$; Fig. 1), but at T48, a clear decrease in zeta potential was observed (from -11.6 to -25.4, $P < 0.05$; Fig. 1). In the absence of organisms, the zeta potential at T48 was higher than at T0 ($P < 0.05$; Fig. 1). The PDI of Ag-NPs showed no significant differences in any experimental conditions (two-away ANOVA, $P > 0.05$; Fig. 1).

The exposure time and the presence of organisms had a significant effect on HDD of CuO-NPs (two-way ANOVA, $P < 0.05$; Fig. 1). At T0, the presence of rotifers and algae resulted in a significant increase in HDD of CuO-NPs (from 419.7–909.5 nm, $P < 0.05$; Fig. 1), but the presence of rotifers and algae did not affect the HDD of NPs at T48 ($P > 0.05$; Fig. 1). The zeta potential of CuO-NPs was affected by the presence of the organisms (two-way ANOVA, $P < 0.05$; Fig. 1) but not by the exposure time ($P > 0.05$). The presence of rotifers and algae increased the zeta potential of CuO-NPs at T0 (from -25.4 to -8.6, $P < 0.05$) and at T48 (from -24.8 to -12.2, $P < 0.05$; Fig. 1). The PDI of CuO-NPs did not change under the experimental conditions (two-way ANOVA, $P > 0.05$; Fig. 1).

Metal dissolution was higher for Ag-NPs than CuO-NPs. At T0 and in the absence of organisms, dissolution of Ag^+ from Ag-NPs was 26.3 % (T0 total Ag = 496.5 ± 2.5 μg L^{-1}, T0 dissolved Ag = 130.6 ± 2.2 μg L^{-1}; Fig S5). The presence of organisms dropped the total amount of silver to 35.4 % and the amount of dissolved Ag^+ to 2.4 % (T0 total Ag = 176.0 ± 11.5 μg L^{-1}, T0 dissolved Ag = 11.9 ± 0.6 μg L^{-1}; Fig S5). At T48 and in the absence of organisms, dissolution of Ag^+ from Ag-NPs was 29.4 % (T48 total Ag = 498.2 ± 1.9 μg L^{-1}, T48 dissolved Ag = 146.0 ± 6.6 μg L^{-1}; Fig S5). The presence of organisms dropped the total amount of Ag to 48.9 % and the amount of dissolved Ag^+ to 4.4 % (T48 total Ag = 242.9 ± 8.5 μg L^{-1}, T48 dissolved Ag = 22.1 ± 0.2 μg L^{-1}; Fig S5).

At T0 and in the absence of organisms, the dissolution of Cu^{2+} from CuO-NPs was 1.2 % (T0 total Cu = 1879.2 ± 95.2 μg L^{-1}, T0 dissolved Cu = 22.2 ± 0.7 μg L^{-1}; Fig S5). The presence of organisms dropped the total amount of Cu to 51.3 % and the amount of dissolved Cu^{2+} to 0.8 % (T0 total Cu = 964.3 ± 52.7 μg L^{-1}, T0 dissolved Cu = 14.9 ± 0.2 μg L^{-1}; Fig S5). At T48 and in the absence of organisms, dissolution of Cu^{2+} from CuO-NPs was 1.0 % (T48 total Cu = 1815.2 ± 114.9 μg L^{-1}, T0 dissolved

Cu = 18.3 ± 0.4 μg L^{-1}; Fig S5). The presence of organisms dropped the total amount of Cu to 33.1 % and the amount of dissolved Cu^{2+} to 0.6 % (T48 total Cu = 622.2 ± 27.6 μg L^{-1}, T0 dissolved Cu = 10.8 ± 0.6 μg L^{-1}; Fig S5).

3.2. Effects of metal nanoparticles and metal ions on rotifer mortality

Both Ag-NPs and CuO-NPs caused mortality of *B. calyciflorus*. In the case of Ag-NPs, the LOEC was 12.8 μg L^{-1} and 100 % mortality was observed at 80 μg L^{-1} (Fig. S3). The LOEC for CuO-NPs was 625 μg L^{-1} (Fig. S3) and 100 % mortality was obtained at 1250 μg L^{-1}. The 50 % lethal effect concentrations of metal ions to rotifers (Ag^{+}, EC_{50} = 4.3 (3.5–4.9) μg L^{-1}; LOEC = 5.1 μg L^{-1}; Cu^{2+}, EC_{50} = 15.2 (14.7–15.7) μg L^{-1}, LOEC = 17.8 μg L^{-1}, Table 1, Fig. S3) were lower than those of respective NPs (Ag-NPs, EC_{50} = 17.5 (14.3–21.9) μg L^{-1}; LOEC = 12.8 μg L^{-1}; CuO-NPs EC_{50} = 600.9 (525.2–700.4) μg L^{-1}, LOEC = 625 μg L^{-1}, Table 1, Fig. S3).

3.3. Effects of metal nanoparticles and metal ions on population growth rate

The exposure of rotifers to Ag-NPs significantly affected the population growth rate; the LOEC of Ag-NPs was 6.25 μg L^{-1} and 100 % of growth inhibition was obtained at 50 μg L^{-1} (Fig. 2). LOEC values for Ag-NPs and Ag^{+} were similar (6.25 μg L^{-1}, Fig. 2). The exposure to CuO-NPs significantly inhibited population growth rate at a LOEC of 62.5 μg L^{-1}, while Cu^{2+} had a LOEC of 50 μg L^{-1} (Fig. 2). The concentrations inhibiting population growth rate by 50 % were lower for ionic forms than for their nanoparticulate forms (CuO-NPs EC_{50} = 83.8 (65.9–101.7) μg L^{-1} vs Cu^{2+} EC_{50} = 50.3 (43.9–56.8) μg L^{-1}, and Ag-NPs EC_{50} = 8.7 (6.2–11.2) μg L^{-1} vs Ag^{+} EC_{50} = 6 (5.1–6.9) μg L^{-1}, Table 1).

3.4. Reactive oxygen species accumulation and plasma membrane damage

The 48 h exposure of rotifers to EC_{10} of each contaminant led to noticeable accumulation of reactive oxygen species (ROS) and damage of plasma membrane comparing to control organisms (Fig. 3). Plasma membrane damage was very pronounced and similar between the four tested contaminants, but ROS accumulation varied with the contaminant (Fig. 3). Cu^{2+} ion led to a higher level of ROS accumulation than CuO-NPs, while both Ag forms led to similar levels of ROS accumulation. The accumulation of ROS and the plasma membrane damage increased with the increase in concentrations of NPs and metal ions (not shown).

Table 1
Effective concentrations for mortality and population growth rate for Ag-NPs, CuO-NPs, Ag^{+} and Cu^{2+}. In brackets are indicated 95 % confidence limits.

Contaminant	Endpoint	LOEC (μg L^{-1})	EC_{10} (μg L^{-1})	EC_{50} (μg L^{-1})
Ag-NPs	Mortality	12.8	5.8 (1.8–8.8)	17.5 (14.3–21.9)
	Population growth rate	6.25	1.7 (0.6–2.9)	8.7 (6.2–11.2)
Ag^{+}	Mortality	5.12	2.9 (1.1–3.7)	4.3 (3.5–4.9)
	Population growth rate	6.25	4.5 (0–9.2)	6.0 (5.1–6.9)
CuO-NPs	Mortality	625	198.2 (112.9–267.6)	600.9 (525.2–700.4)
	Population growth rate	62.5	46.9 (27.6–66.1)	83.8 (65.9–101.7)
Cu^{2+}	Mortality	15.22	12.8 (11.9–13.5)	15.2 (14.7–15.7)
	Population growth rate	50	35 (8.0–62.0)	50.3 (43.9–56.8)

3.5. Transgenerational effects of metal nanoparticles and metal ions

In the control, the population growth rate of rotifers was higher than 0.55 d^{-1} and no evidence of parental mortality. The exposure to EC_{50} of each contaminant led to a significant inhibition ($P < 0.05$) in the population growth rate (Fig. 4). Mortality was only observed under Cu^{2+} exposure showing 1 death parent. At F1, the population growth rate was significantly inhibited ($P < 0.05$) by exposure to Ag-NPs and Cu^{2+}. Mortality at F1 was observed after exposure to Cu^{2+} and Ag-NPs, and in rotifers pre-exposed to Ag^{+}. At F2 generation, only the rotifers exposed to Ag-NPs had a decreased population growth rate. Parental mortality was low but still occurred in rotifers under exposure to Cu^{2+} and in those pre-exposed to Ag-NPs. At F3 and F4 generations, the contaminants did not affect the rotifer growth rates. However, parental mortality occurred in rotifers at F3 under Cu^{2+} exposure, and at F4 under exposure to CuO-NPs, pre-exposed to Ag-NPs or Ag^{+}. The population growth rate of rotifers pre-exposed to the contaminants from F1 to F4 generations did not differ from control (one-way ANOVA, $P < 0.05$; Fig. 4).

Although recovery of population growth rate was observed at F3 and F4, rotifer development delays occurred in at least one replicate per treatment and were intrinsically connected to parental mortality observed (Fig. S4).

At F0 generation, ROS accumulation and plasma membrane damage were clearly visible in all treatments apart from the control (Fig. 5). At F4 generation, ROS accumulation and plasma membrane damage slightly increased after exposure to Ag-NPs and Ag^{+}, while decreased after exposure to CuO-NPs and Cu^{2+}. At F4, the rotifers pre-exposed to Ag^{+} and Cu^{2+} showed ROS accumulation and plasma membrane damage, while the rotifers pre-exposed to Ag-NPs and CuO-NPs showed very faint ROS accumulation and had the least plasma membrane damage (Fig. 5).

4. Discussion

This study showed that metal nanoparticles and their ionic forms might be toxic to freshwater populations of *B. calyciflorus*, leading to mortality and reducing population growth rate. Both lethal and sublethal effects of Ag-NPs were more pronounced than those of CuO-NPs (effects on population growth rate: EC_{50} = 8.7 μg L^{-1} for Ag-NPs vs 83.8 μg L^{-1} for CuO-NPs). An opposite trend was observed for leaf decomposition by aquatic microbes where CuO-NPs had a more pronounced impact than Ag-NPs (Pradhan et al., 2011). Both Ag-NPs and CuO-NPs showed a tendency to be less toxic to rotifers than their respective ionic forms. A less pronounced effect of metal nanoparticles on aquatic organisms compared to their ionic counterparts have been reported (Pradhan et al., 2011; Garner et al., 2015; Batista et al., 2017).

In our study, the rotifers and algae removed a higher amount of Ag-NPs from the medium when compared to Ag^{+} (Fig. S2), which might have resulted from a higher accumulation and/or adsorption and/or lower release of nanoparticles by the rotifers. A higher Ag^{+} uptake compared to its nanoparticle form was reported by *D. magna* (Ribeiro at al., 2017), which could be due to the uptake of Ag^{+} by cells through ion transporters (Luoma, 2008), increasing the entry of Ag^{+} more than of Ag-NPs. On the other hand, the uptake of metal NPs has been related to endocytosis processes (Pan and Wang, 2004; Ribeiro et al., 2017) that can be influenced by hydrodynamic size of nanoparticles. Considering the characteristics of Ag^{+} and Ag-NPs, a lower uptake rate of Ag-NPs associated with a lower elimination rate of Ag-NPs might have been responsible for the lower amount of silver in media in the presence of the organisms. This pattern was also observed in *D. magna*, in which depuration of Ag-NPs was lower than that of Ag^{+} due to their larger size (Ribeiro et al., 2017). In our study, the presence of rotifers and algae led to a reduction in the hydrodynamic size of Ag-NPs in the medium, possibly due to accumulation and/or adsorption of larger particles to the organisms; this was further supported by the decrease in zeta potential of Ag-NPs in the presence of the organisms after 48 h of exposure (-11.6

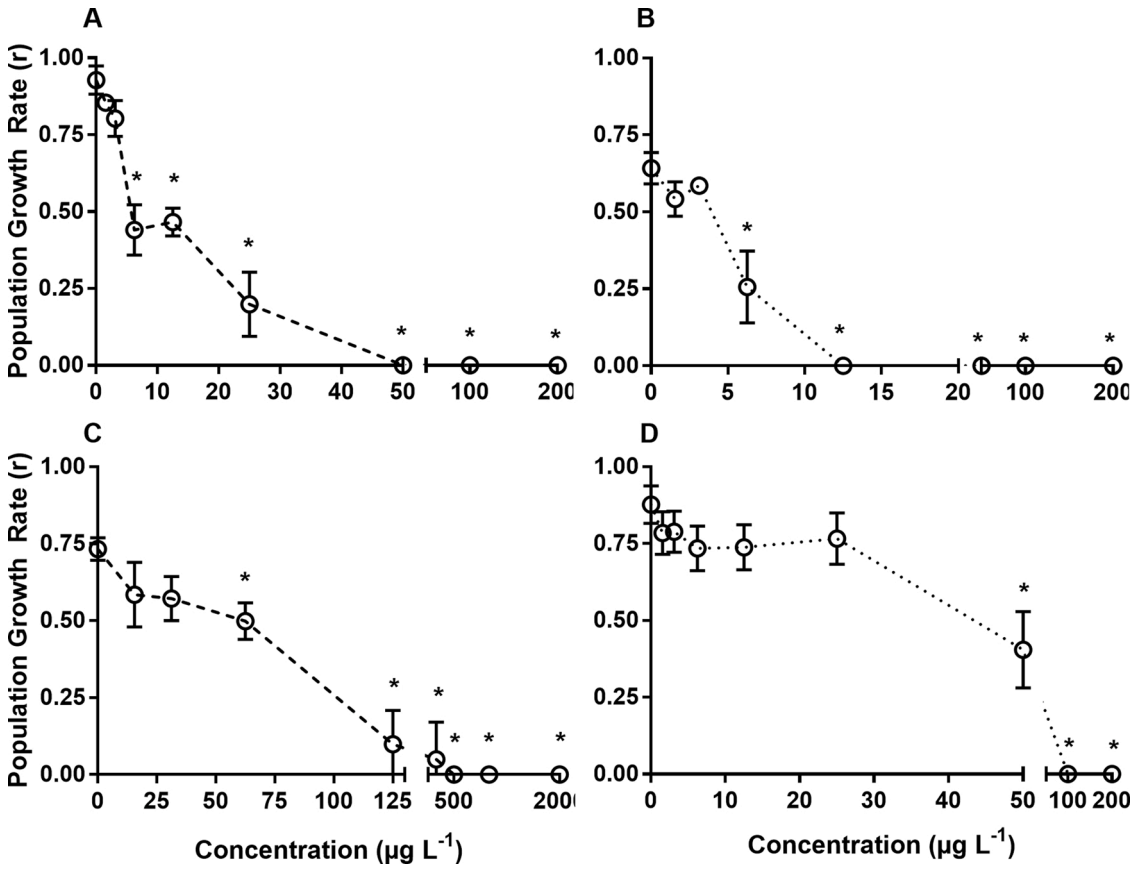

Fig. 2. Population growth rate of *B. calyciflorus* exposed to increasing concentrations of: A) Ag-NPs, B) Ag^{+}, C) CuO-NPs and D) Cu^{2+}. Values are mean ± standard error of the mean, n = 8. * significantly different from the control (One-way ANOVA, Dunnet's test $P \leq 0.05$).

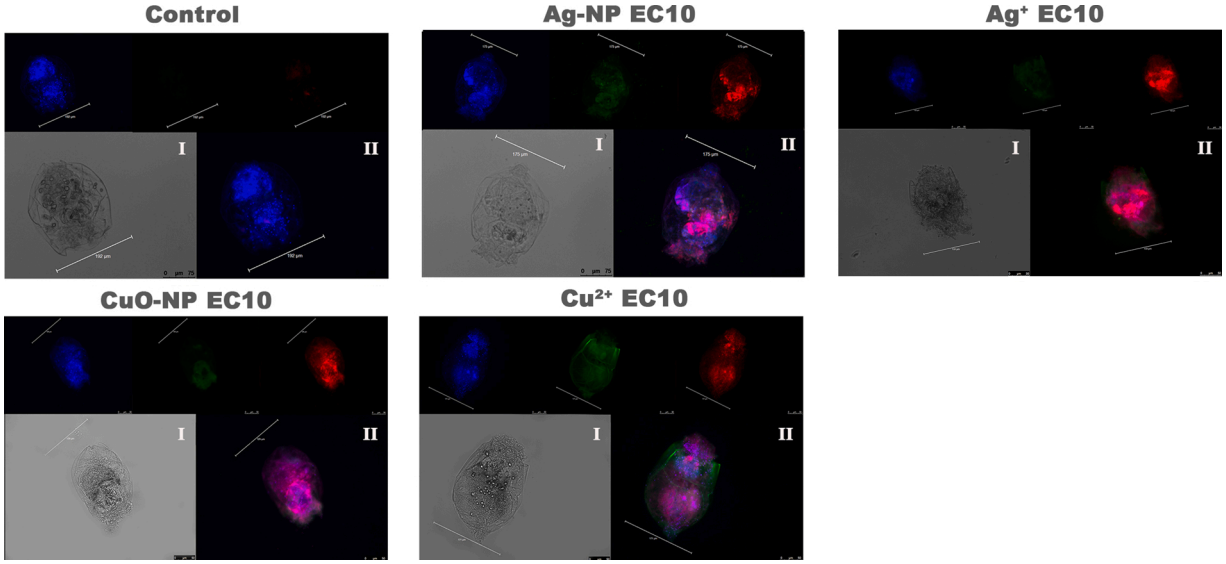

Fig. 3. Epifluorescence microscopy images of rotifers exposed to EC_{10} of Ag-NPs, Ag^{+}, CuO-NPs and Cu^{2+}. Blue fluorescence shows co-localized nuclei after staining with anti-fading reagent containing DAPI, green fluorescence indicates the intracellular accumulation of reactive oxygen species (ROS) after staining with CM-H_2DCFDA, and red fluorescence indicates plasma membrane damage after staining with propidium iodide. I) bright field images; II) composite images with the 3 stains.

mV to -24.6 mV). If rotifers accumulate and/or adsorb NPs, the possibility of trophic transfer of Ag-NPs cannot be discarded. When looking at EC values for the effects of Ag-NPs on *B. calyciflorus* (EC_{10} = 5.8 µg L^{-1}) compared to the detected concentrations in the environment (0.3–6.6 ng L^{-1}; Peters et al., 2018), an assumption of low environmental risk might be assumed. However, a worst scenario can be envisaged when considering the predicted environmental concentrations that are much closer to EC values (up to 0.6 µg L^{-1}; Musee, 2011). Nevertheless, the higher accumulation in organisms and possible release of Ag ions indicate an unpredictable contribution of Ag-NPs to the increase in total

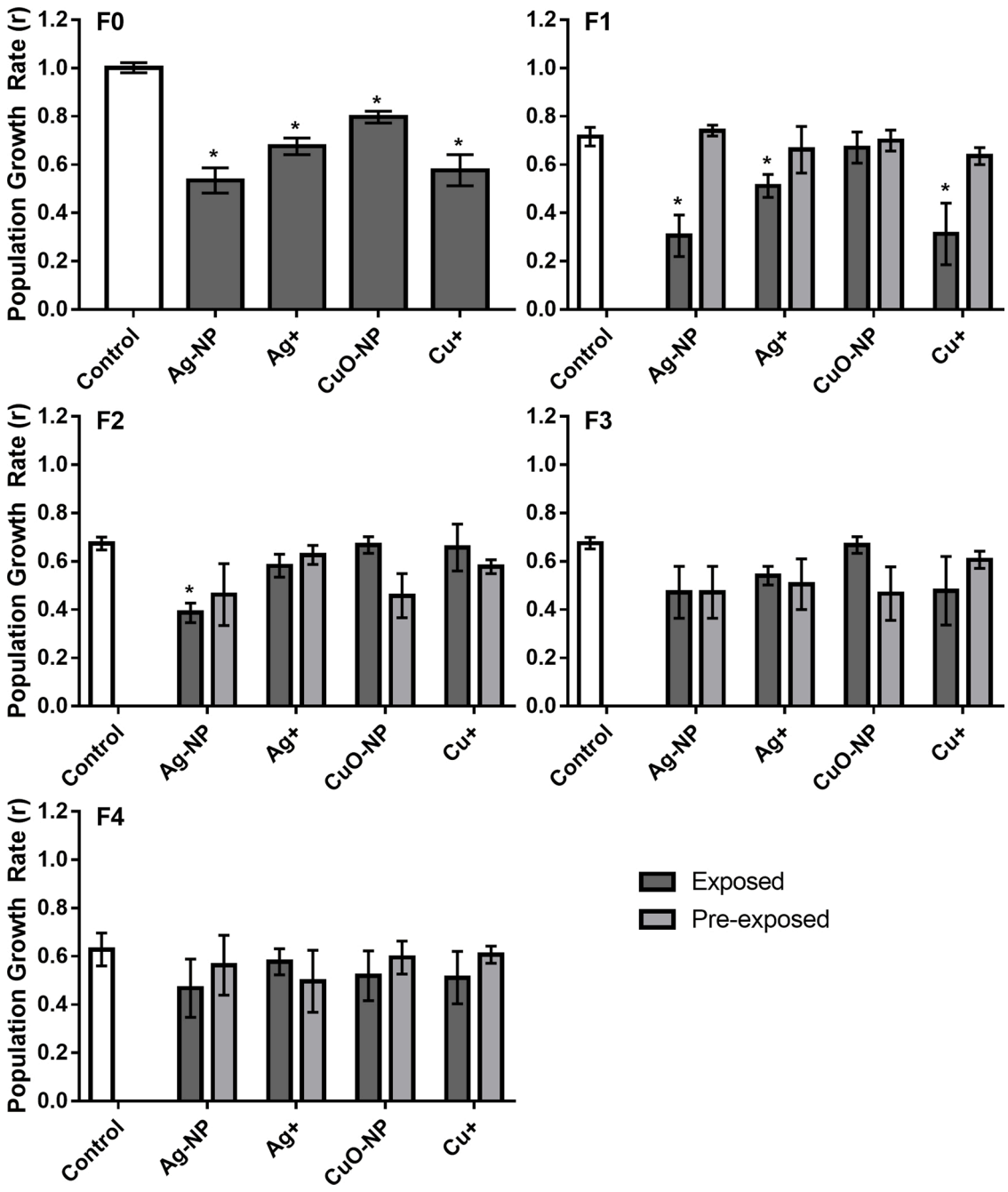

Fig. 4. Effects of Ag-NPs, CuO-NPs, Ag^{+} and Cu^{2+} and recovery of rotifers at F0, F1, F2, F3 and F4 in terms of population growth rate. Values are mean ± standard error, n = 8. * statistical different from the control (one-way ANOVA; $P \leq 0.05$).

silver in aquatic environments with potential impacts in the longer term.

In our study, CuO-NPs were less toxic than Cu^{2+} to rotifers as depicted by the effects on mortality and population growth rate. Our results are in alignment with those from *D. magna* showing stronger effects of Cu^{2+} ($EC_{50} = 22\ \mu g\ L^{-1}$) than of CuO-NPs ($EC_{50} = 1041\ \mu g\ L^{-1}$) on reproduction (Adam et al., 2015), and are in alignment with the general trend observed through species sensitivity distribution of CuO-NPs and their ionic forms for aquatic organisms (Garner et al., 2015). The entry of Cu^{2+} into cells through the ion transporters (Luoma, 2008) may contribute to explain the high toxicity of metal ions. Although Cu^{2+} released from CuO-NPs might contribute to the overall CuO-NPs toxicity, dissolution data showed that contribution of ionic Cu was low, in our study. Agglomeration and particle stability are postulated to interfere with the uptake, availability and depuration of CuO-NPs from organisms and, therefore, affecting toxicity (Adam et al., 2015). Indeed, this was observed in our study, where aggregation of CuO-NPs increased along time. This can be the result of interactions between components of the medium and the nanoparticles and/or processes of self-agglomeration (Pradhan et al., 2014), explaining the increase in the hydrodynamic size and the decrease in stability. Surprisingly, higher accumulation of Cu in rotifers occurred after exposure to CuO-NPs than to Cu^{2+} (Fig. S2), suggesting that rotifers can uptake CuO-NPs at a higher rate than Cu^{2+} and/or showing lower elimination rate as discussed for Ag-NPs. A similar trend was found in *D. magna*, in which copper body burden was 2.8–42 times higher under exposure to CuO-NPs than to Cu^{2+} (Muna et al., 2017). Despite the apparent higher removal of CuO-NPs by rotifers and algae, the toxicity was lower than the one exhibited by Cu^{2+}. Comparison of CuO-NPs EC values on

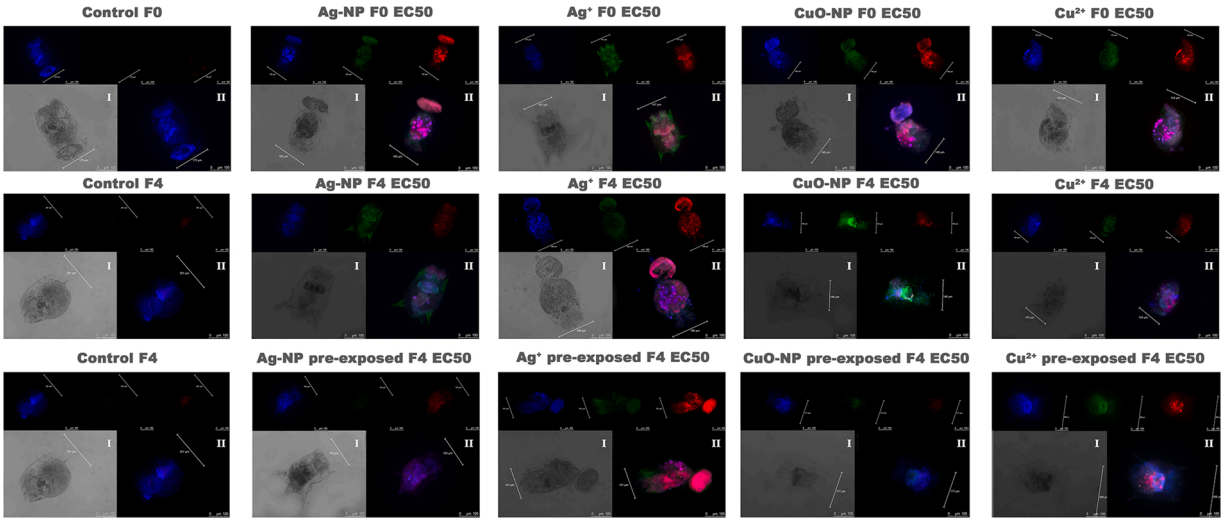

Fig. 5. Epifluorescence microscopy images of F0 and F4 generation of rotifers exposed or pre-exposed to EC_{50} of Ag-NPs, Ag^{+}, CuO-NPs and Cu^{2+}. Blue fluorescence shows co-localized nuclei after staining with anti-fading reagent containing DAPI, green fluorescence indicates the intracellular accumulation of reactive oxygen species (ROS) after staining with oxidative stress indicator CM-H_2DCFDA, and red fluorescence indicates plasma membrane damage after staining with propidium iodide. I) bright field images; II) composite images with all 3 stains.

B. calyciflorus (population growth rate $EC_{10} = 46.9\ \mu g\ L^{-1}$) to environmental concentrations is difficult because few data are available. However, in industrial wastewaters CuO-NPs can exceed 100 mg L^{-1} (Huang et al., 2006), making our conclusions relevant.

There is still a controversy on the origin of NPs toxicity: some studies indicate that the toxicity of CuO-NPs is the consequence of the dissolution of Cu^{2+} from the nanoparticles (Jo et al., 2012; Adam et al., 2015), while others claim the occurrence of nanoparticle-specific effects (Kasemets et al., 2009; Manusadžianas et al., 2012). The same is applicable to Ag-NPs where some studies show a similar toxicity mechanism to Ag^{+} through the disruption of Na regulation (Kwok et al., 2016), while others show that due to low dissolution of metal ions from the Ag-NPs, the toxicity is attributed mainly to their nano form (Batista et al., 2017; Clark et al., 2018). The latter hypothesis is supported by results from aquatic microbes, where proteomic profiles of microbes exposed to Ag-NPs and Ag^{+} largely differ, suggesting distinct cellular targets and mechanisms of toxicity (Barros et al., 2020). In our study, dissolution of Ag ions from Ag-NPs in the medium without organisms was low and was even lower in the presence of organisms, reaching concentrations near to Ag^{+} EC_{50} values. This suggests that Ag^{+} released from Ag-NPs cannot explain the observed toxicity, so nanoparticle-specific effects might have occurred. When it comes to CuO-NPs, dissolved Cu^{2+} (c.a. 1% of total copper dissolved in medium) was even lower than Ag^{+}. Therefore, dissolved Cu^{2+} appeared to have a minor role in the observed toxicity.

In our study, the toxicity of all metal forms (Ag-NP, Ag^{+}, CuO-NP and Cu^{2+}) was associated with ROS accumulation and plasma membrane damage. The connection between Ag^{+} and Ag-NPs toxicity and their ability to induce the accumulation of ROS has been widely reported (Kwok et al., 2016; AshaRani et al., 2009; Dąbrowska-Bouta et al., 2019; Khan et al., 2019), including in human cell lines, in which the toxicity of Ag-NPs has been mainly attributed to the induction of oxidative stress, protein and DNA damage, and apoptotic death (Arora et al., 2008; Hsin et al., 2008; Kim et al., 2009). Similarly, many studies claim a similar connection for CuO-NPs (Karlsson et al., 2009; Petersen and Nelson, 2010; Chang et al., 2012; Pradhan et al., 2015). This hypothesis was supported by our study with a strong accumulation of ROS and visible membrane damage at the EC_{10} concentration of both nanoparticles. Accumulation of ROS was more pronounced for Cu^{2+} than CuO-NPs, regardless the higher retention of CuO-NPs by rotifers, further suggesting that CuO-NPs had a specific toxicity pathway independent of the dissolution of Cu^{2+} and its internalization by rotifers.

The exposure of rotifers to Ag-NPs and Ag^{+} across generations showed a recovery of rotifer population growth rate. Specifically, long-term exposure to Ag^{+} led to the recovery at the F1 generation, while the recovery from Ag-NPs only occurred at the F3 generation. Although the population growth rate recovered, a delay in the reproduction was observed, probably indicating that the animal fitness was affected. A similar result was found in *C. elegans* with multigenerational exposure to sub-lethal concentrations of Ag-NPs resulting in a higher tolerance revealed by fertility and fecundity, but a delay in development and somatic growth (Rossbach et al., 2019). Moreover, rotifer populations continuously exposed to Ag-NPs and Ag^{+} were under stress, as shown by ROS accumulation and plasma membrane damage in F4 populations. Therefore, we can infer that rotifer offspring resulting from the contaminated F0 generation exhibited signs of stress even after four generations. However, F4 rotifers pre-exposed to Ag-NPs and then released from the contaminant showed signs of recovery with lower accumulation of ROS and lower level of plasma membrane damage. In our study, the population growth rate recovery might be related to the selection of more resistant genotypes rather than a recovery of the animal fitness. Similarly, in *C. elegans*, Ag^{+} and Ag-NPs caused differences in size and lifespan in the F2 generation, and effects persisted in the pre-exposed generations after the removal of contaminants (Schultz et al., 2016). On the other hand, Mendes et al. (2018) showed silver multigeneration exposure of *Folsomia candida* resulted in increased effects after 4 generations. The removal from contaminants led to reproduction recovery in terms of number of juveniles, but arthropods had a smaller size than those of control (Mendes et al., 2018). These results support the idea that effects caused by Ag-NPs and Ag^{+} may persist in different organisms along time.

The toxicity of CuO-NPs, assessed by EC_{50}, slightly decreased along the rotifer generations continuously exposed to the toxicant; this can be explained by the low stability of the nanoparticles with time that might have influenced their concentration and bioavailability. A full recovery in the population growth rate was observed for CuO-NPs at F1 and Cu^{2+} at F2 and further generations. The rotifers, released from the contaminants at F1, recovered regardless the Cu form. As observed for Ag-NPs, we found a delay in reproduction even when population growth rate had already recovered. Intracellular ROS accumulation and plasma membrane damage was still observed in the rotifers exposed either to CuO-NPs or Cu^{2+} till the F4 generation even though population growth rate

recovered. However, the rotifers pre-exposed to CuO-NPs showed signs of recovery at F4, with lower accumulation of ROS and less plasma membrane damage. CuO-NPs seemed to show a less pronounced transgenerational effects than Cu^{2+}, supporting different mechanisms of toxicity. Yu et al. (2013) showed clear transgenerational effects of metals (Cu, Cd, Pb and Zn) in *C. elegans*, with a greater impact on the behaviour and growth inhibition for the progeny at F1 generation. This suggests that the parental exposure can increase the sensitivity of future generations to toxicants. Multigenerational effects of CuO-NMs and $CuCl_2$ on the soil invertebrate *Enchytraeus crypticus* showed no change in sensitivity to CuO-NMs but increased toxicity at EC_{10}, with organisms recovering from contamination once transferred to clean medium (Bicho et al., 2017), as observed in our study. Meanwhile, the multigeneration exposure to $CuCl_2$ reduced the toxic effects at EC_{10} and EC_{50} concentrations but the transfer to clean medium increased the effects (Bicho et al., 2017). These studies support the idea that different mechanisms of action are responsible for the toxicity of CuO-NPs and Cu^{2+} and also show that different endpoints and populations may have different responses across generations, with some populations showing recovery through generations and others an increased sensitivity.

5. Conclusions

The toxicity of metal ions and metal NPs towards the rotifer *B. calyciflorus* was translated in mortality and inhibition of population growth rate. Dissolution data showed that the effects of metal NPs were nanoparticle specific with CuO-NPs showing very low dissolution in our study. Oxidative stress was observed in rotifers exposed to low effect concentrations (e.g., EC_{10}) of both ions and NPs. Contrarily to our predictions, rotifers exposed to ions and NPs were able to fully recover its population growth rate at F3, but, in certain cases, there was a delay in their reproduction. This can be partially explained by ROS accumulation and plasma membrane damage. Rotifer populations showed signs of resilience to both metal ions and NPs, but the higher accumulation of metal NPs than metal ions might be a risk in the longer term. Overall, our study represents a step forward in exploring toxicant effects beyond F0 generation to better assess the risk of metallic nanoparticles and their ionic counterparts in freshwaters.

CRediT authorship contribution statement

Nuno Martins: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft. **Arunava Pradhan:** Conceptualization, Methodology, Supervision, Investigation, Writing - review & editing. **Cláudia Pascoal:** Conceptualization, Methodology, Supervision, Writing - review & editing. **Fernanda Cássio:** Conceptualization, Methodology, Supervision, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105652.

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