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Surface-to-Volume Ratio Affects the Toxicity of Nanoinks in Daphnids

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Abstract: The Organization for Economic Co-operation and Development (OECD) has set widely used guidelines that are used as a standardized approach for assessing toxicity in a number of species. Given that various studies use different experimental setups, it is difficult to compare findings across them as a result of the lack of a universally used setup in nano-ecotoxicology. For freshwater species, *Daphnia magna*, a commonly used filter feeding crustacean, can generate significant molecular information in response to pollutant exposure. One factor that has an effect in toxicity induced from nanomaterials in daphnids is the surface-to-volume ratio of the exposure vessels; however, there is limited information available about its impact on the observed effect of exposure. In this study, daphnids were exposed to silver nanoparticle ink in falcon tubes and Petri dishes for 24 h. Toxicity curves revealed differences in the observed mortality of daphnids, with animals exposed in Petri dishes displaying significantly higher mortality. Differences in the activities of a number of key enzymes involved in the catabolism of macromolecules and phosphate were also observed across the exposure setups, indicating possible differences in the toxicity mechanism of silver nano-ink. Understanding the impact of factors relevant to experimental setups in ecotoxicology can increase the reproducibility of testing, and also reduce experimental costs, time, generated waste, and daphnids used in research.

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1. Introduction

Nanoparticles (NPs) are currently present in an increasing number of products used in our daily lives, and their commercial applications are increasing every year. The market of NPs reached more than USD 2 billion in the U.S. alone by 2021 [1], with most NPs being used in the industrial, medical, and food sectors [2]. Titanium dioxide, silica, and carbon nanotubes are among the most commonly used products, of which are there many [3]. With more NPs being applied annually, more and more products find their way into the terrestrial and aquatic environment as emerging pollutants [4], and knowledge of their true impact on the environment is severely lacking. Crucial information on the toxicity of NPs and their environmental impact on the aquatic environment can be obtained with toxicity testing using bioindicator species, e.g., crustaceans such as *Daphnia magna* (*D. magna*), mussels such as *Mytilus galloprovincialis* [5], and other species that are less advanced in their evolutionary development, which are used as alternative approaches to replace the use of higher animals in research [6,7]. The planktonic crustacean *D. magna* is one of the most commonly used bioindicator species in toxicity testing and environmental monitoring [8]. Other daphnia species are also used in both acute and chronic toxicity testing [9,10]. A variety of biomarkers can be used to assess the impact of pollutant exposure

to the physiology of exposed individuals [10–13], providing the means to detect any kind of adverse effect (e.g., genotoxicity and cytotoxicity) on aquatic life.

The negative impact of metallic NPs, such as silver NPs, on in vivo and in vitro systems has been well documented in multiple studies [14–22]. In the available literature, there is a plethora of evidence that illustrates the adverse effects of silver nanoparticles (AgNPs) on aquatic life. AgNPs have been documented to generate oxidative damage and tissue damage to fish [23,24], which can impair the survival and growth of invertebrates such as *D. magna*, as well as other aquatic life, including algae [25–27]. However, it is worth noting that in the available literature, studies on the ecotoxicological impact of AgNPs have shown inconsistent results [28–32], where the EC_{50} values of AgNPs vary significantly across studies due to the inconsistency of experimental designs across laboratories. Therefore, this makes the evaluation of the impact of AgNPs in the ecotoxicological context very difficult. One possible explanation for such discrepancies is the multitude of NP characteristics that impact its toxicity (e.g., size [33], coating [34], and shape [35]); however, there is little-to-no available information on the fate of NPs in the test system. By increasing the focus on the test system itself, we may be able to better understand the mechanistic aspects of nano-toxicity and eliminate the way in which factors which are unrelated to pollutants (NPs) themselves impact the study.

Phenotypic and enzyme markers of physiology were used to evaluate the impact of S:V on the physiology of daphnids following exposure to nano-inks. In relation to NP toxicity testing, this study demonstrates that the traditionally varying experimental setup of toxicity exposures can generate wildly divergent results due to differences in the S:V of exposure vessels alone. Furthermore, we observed that S:V can alter the certain endpoints of animal physiology, even in the absence of nano-ink pollutants.

2. Results

Shallow vessels such as Petri dishes have a high S:V ratio, while tall or deeper vessels such as Falcon tubes have a lower S:V ratio. The impact of S:V was investigated here in the context of how it affected mortality and biochemical and phenotypic markers of physiology of daphnids in response to exposure to silver nano-inks.

### 2.1. Acute Exposure to Silver Nanoparticle Ink in Different Vessels

The toxicity of silver nano-ink was assessed in three exposure conditions: falcon tubes with 50 mL of media, and Petri dishes with 50 mL or 100 mL of OECD media. Toxicity curves were plotted and the EC values were calculated in a similar order of magnitude; however, the falcon tube had the highest EC_{50} value of 4.33 µL of nano-ink/L, whereas the EC_{50} values of the Petri dish were 1.443 µL/L and 1.182 µL nano-ink/L for 50 and 100 mL of media, respectively (Figure 1). EC_1 values of the toxicity curves were calculated and used for the selection of exposure concentrations for daphnids (EC_1^{Falcon}: 0.561 µL/L, EC_1^{Petri}: 0.77 µL/L). The non-lethal exposure concentration selected was used for the assessment of silver nano-ink exposure to the activity of key enzymes in daphnid metabolism. To reduce the number of experimental variables throughout the exposures, the 50 mL Petri dish was selected as a comparison vessel to the 50 mL Falcon tube. The decision to employ this approach was based on the intention of avoiding any alterations to other vessel parameters, such as volume and animals/mL (the crowding of daphnids) in the vessel comparison, in order to eliminate their potential impact on silver nano-ink toxicity.
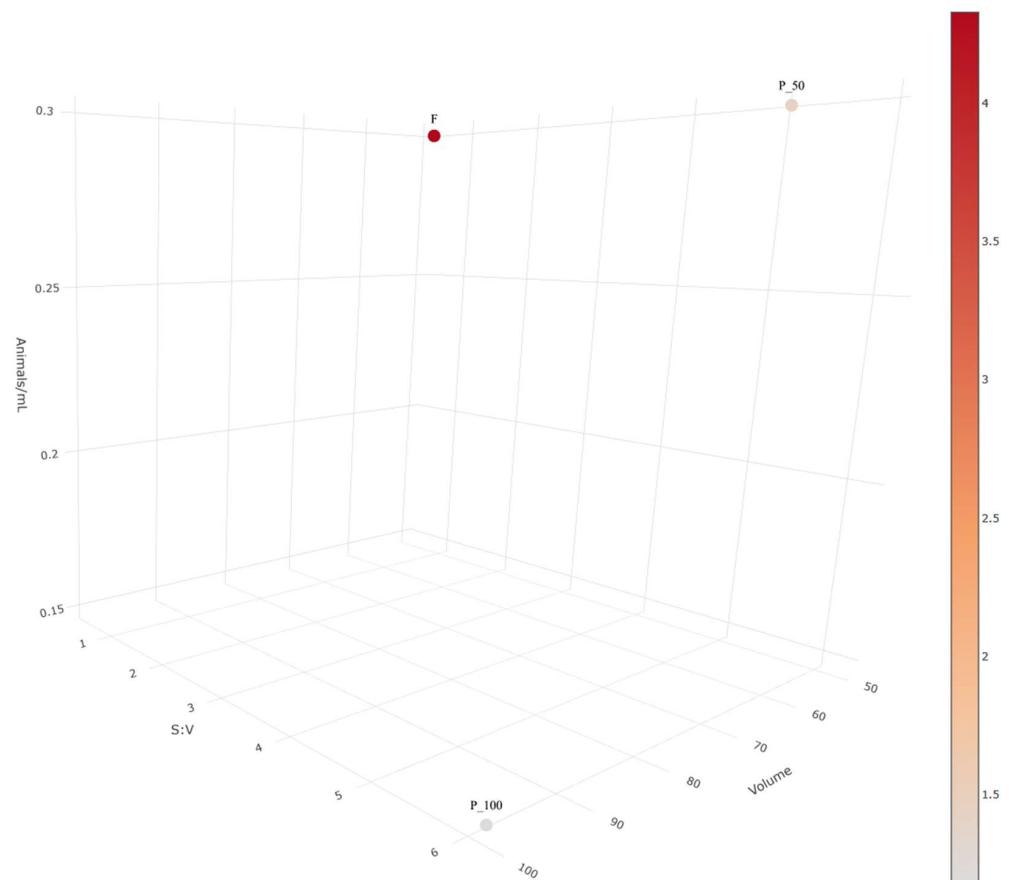
Figure 1. The impact of S:V, volume, and animals/mL on the toxicity of silver nanoparticle ink. EC_{50} values are expressed in μL/L in the color scale. An interactive version is provided in Supplementary Materials.

2.2. Acute Biochemical Responses to Nanomaterials

Exposure to the silver nano-ink in the falcon tube and the Petri dish at a constant volume of 50 mL triggered different responses in the activity of enzymes (Figure 2). Exposure in the falcon tube resulted in a different profile of enzyme activities in the exposed individuals, when compared to the enzyme activities of daphnids in the Petri dish exposure condition. For the Falcon tube exposure condition, the exposure of daphnids in 0.5 μL of nano-ink/L generated statistically significant changes in the activity of β-galactosidase (BGAL) and lactate dehydrogenase (LDH) with an observed increase of 8.84% and a decrease of 8.09%. In the exposure concentration of 1 μL of silver nano-ink/L, the activity of BGAL, peptidase (PEP), acidic phosphatase (ACP), and alkaline phosphatase (ALP) increased by 12.12%, 4.95%, 29.27%, and 41.69%, respectively. A dose-depended effect can be observed for β-galactosidase, ACP, ALP, and reduced thiols in the Falcon tube exposure condition. In the Petri dish exposure condition of 0.5 μL silver nano-ink/L, statistically significant differences were observed for the activity of LDH (62.66%). Interestingly, this was the largest change in enzyme activity recorded in this study. For the 1 μL/L silver nano-ink exposure condition, a significant increase in LDH activity (56.17%) was observed, followed by a significant decrease in the enzyme activity of peptidase (−11.25%), ACP (−33.45%), and ALP (−20.43%). Dose-dependent enzyme activity changes were also observed for LDH, ACP, and ALP in a concentration-dependent profile.
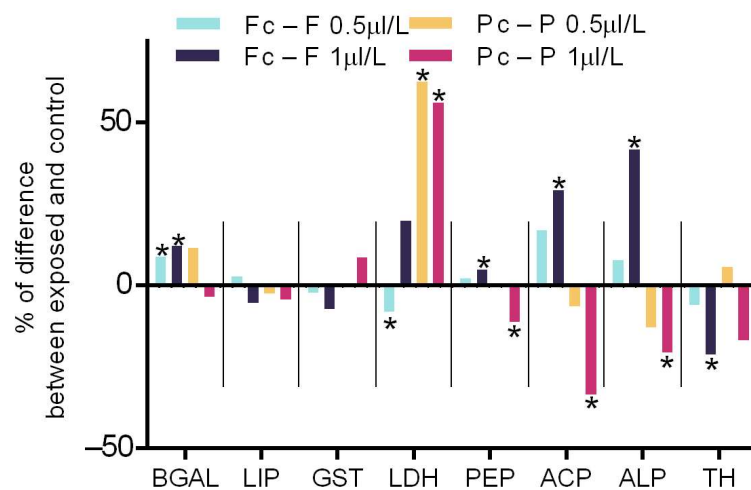
Figure 2. The impact of silver nano-ink in enzyme activity in relation to S:V difference. The data represent the percentage of difference in the enzyme activity of daphnids exposed to silver nano-ink compared to their unexposed control ($N = 4$). The Mann Whitney U test (*) denotes significant differences between the two conditions. Abbreviations: Fc: falcon control, F: falcon exposed, Pc: Petri control, P: Petri exposed, BGAL: β-galactosidase, LIP: lipase, GST: glutathione-S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, TH: reduced thiols.

The impact of S:V on daphnids in the absence of silver nano-ink was assessed using an array of biochemical assays to compare the two control conditions (Figure 3). The unexposed controls for the Petri resulted in statistically significant changes in the activity of BGAL (+5.55%), LIP (−11.1%), and ALP (+27.6%), and a non-statistically significant decreasing trend for LDH, ACP, and reduced thiols compared to the control in the falcon tubes.
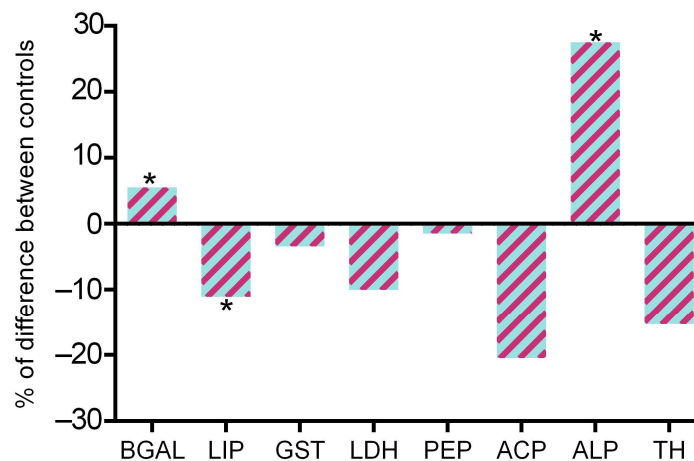

Figure 3. The impact of SV on unexposed controls. Comparisons of enzyme activity between the unexposed controls of the Falcon tube and the Petri dish. The data represent the average ($N = 4$) replicates for each condition. The Mann Whitney U test (*) denotes significant differences between the two conditions. Abbreviations: BGAL: β-galactosidase, LIP: lipase, GST: glutathione-S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, TH: reduced thiols.

2.3. Feeding and Microscope Images

The impact of nano-inks was evaluated with non-invasive tests on the feeding rate of exposed daphnids. The feeding rate is a phenotypic endpoint used in multiple studies to evaluate the physiology of daphnids [36–38]. The fluorescent microparticles used were

selected because they have a mean particle size of 2.0 μm and *D. magna* feeds non-selectively on a wide range of particles with sizes ranging from 1 to 50 μm, which would allow microparticles to be tracked with fluorescence microscopy [39]. In addition, toxicity testing revealed that the microparticle concentration used for the assay was not toxic to daphnids. The feeding rate of daphnids exposed in Petri dishes was significantly more impacted compared to daphnids exposed in Falcon tubes. Furthermore, differences in the feeding rate when compared to their respective controls were higher for daphnids exposed in Petri dishes (Figure 4).
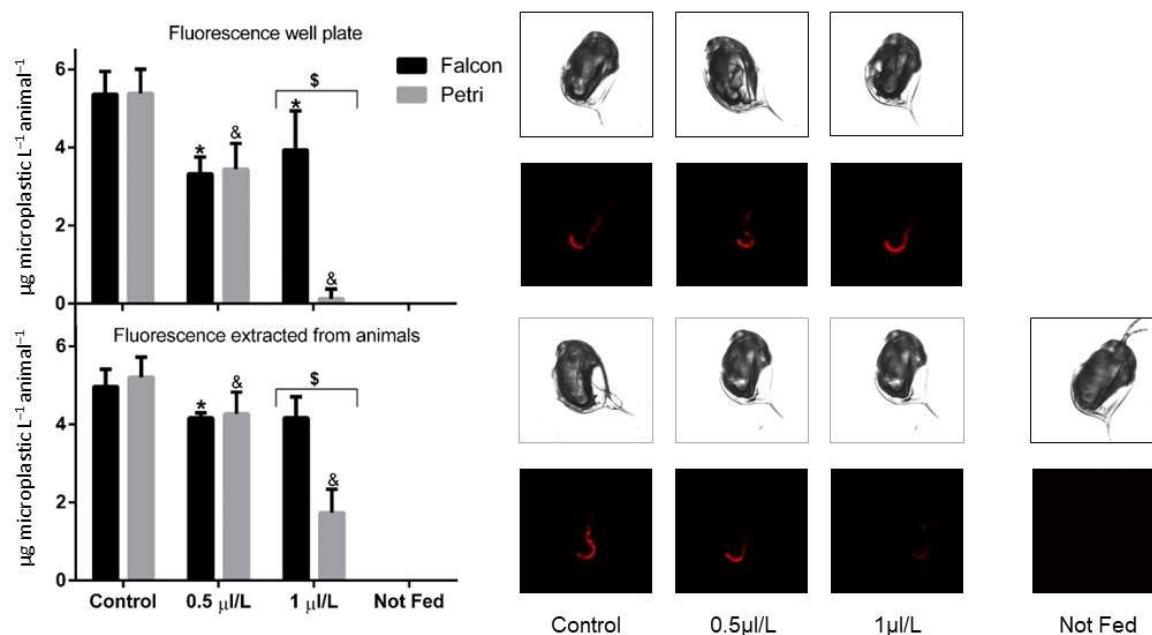
Figure 4. The impact of silver nano-ink exposure on the feeding rate of daphnids. The ingestion of fluorescent microparticles was visualized using bright field and fluorescence microscopy (panel A). The feeding rate was quantified by the fluorescence of ingested microparticles in the incubation media or extracted from daphnids after 30 min (panel B). The data represent the mean ± standard deviations ($N = 4$). The *, & symbol indicates statistically significant differences found by the Mann Whitney U test, from the unexposed control to silver nano-ink, and from 0.5 μL/L to 1 μL/L, respectively. The '$' on the bracket symbol indicates statistically significant differences between the same concentration of silver nano-ink in the two different exposure vessels. "Not Fed" displays the fluorescence measured in daphnids that were not incubated with microplastic before the start of the start of the feeding assay to acquire any background fluorescence.

3. Discussion

In this study, we investigated the impact of vessel S:V in the context of nanotoxicology by exposing daphnids to AgNPs in two vessels with different S:V ratios. Most laboratory studies that use NPs do not use the same, or sometimes even similar, vessels for the toxicity assays, which can create a discrepancy in the results. In any experimental setup for toxicity testing for NPs, it is mandatory that the physicochemical properties are considered. NPs are never fully dissolved in solution; hence, an NP solution is characterized as a colloidal suspension. Consequently, the S:V of the exposure vessel can change the toxicity of pollutants that create colloidal suspensions [40] because it could influence the availability of NPs to exposed individuals [31,41]. According to the available literature [42], S:V can impact several parameters of daphnid physiology, such as mortality and feeding rate; however, information on the true impact of S:V on NP toxicity remains extremely scarce. To the best of our knowledge, there are only two studies that have investigated the impact of S:V in a toxicological context [42,43]. In a study by Grintzalis et al. [42], the impact of S:V was investigated under the prism of a non-NP pollutant. The S:V range that was

investigated in different vessels was much smaller (0.12–0.90 cm^2 mL^{-1}) than the range used in this study (Falcon tube S:V: 0.493 cm^2 mL^{-1} and Petri dish S:V: 5.089 cm^2 mL^{-1}). According to Grintzalis et al. [42], S:V, as a factor in the experimental design, did not impact the toxicity of CdCl$_2$ to daphnids, while our findings indicate that S:V has an insoluble contaminant impact on the daphnids. Baumman et al. [43] reported that S:V impacted the toxicity of AgNPs in a miniaturized toxicity approach compared to its control. However, the experimental design in the study by Baumman et al. resulted in changes in the daphnid crowding inside the exposure vessels, which could result in additional factors besides the vessel S:V impacting the observed effect. The changes to daphnid crowding could have also influenced the potential impact of S:V as there is no available information on the synergistic effects of experimental design factors, such as S:V, volume, and daphnid crowding, on NP exposure. Furthermore, the S:V vessel differences that were investigated in the study by Baumman et al. were smaller (2.08–3.50 cm^2 mL^{-1}) compared to the difference of S:V of vessels investigated in this study. Furthermore, the uniformity of volume, the number of daphnid individuals per replicate, and the vessel material were maintained across all tested conditions. Additionally, the sole experimental parameter that changed across the exposure vessels was the S:V.

One plausible explanation for the observed impact of S:V is that daphnids undergo changes in the activity of many key enzymes merely by being placed in a flattened or shallow exposure vessel with a small water column, such as a Petri dish, even without presence of a pollutant (Figure 3). The reason for these key changes could be attributed to the restriction of the diel vertical migration of daphnids [39]; however, no available information exists in the literature on the impact of restricting diel migration in daphnids. What is known so far is that diel vertical migration is related to the presence of predators, and that chemical cues such as kairomones are responsible for "alerting" changes in the daphnid's environment [44].

The profile of enzyme activity for individuals in the two exposed conditions was divergent when they were compared with their respective controls, although there were a few similarities as well. According to Choi et al. [45], pollutants with similar modes of toxicity have similar profiles of gene expression, and, for AgNPs, it is known to be a case for aquatic organisms such as *Danio rerio* [45], as well as other Cladocera species such as *Chydorus sphaericus* [46] and *Daphnia magna* [47]. According to the available literature [47], AgNPs and Ag$^+$ generate different gene expression patterns in exposed daphnids. In a solution that contains AgNPs, daphnids are exposed to NPs as well as Ag$^+$ ions because of the dissolution [48]; therefore, one hypothesis states that daphnids exposed in Falcon tubes may "interact" more with silver in its ionic form rather than silver NPs due to the possible NP sedimentation [49]. Our findings support this hypothesis, as the observed enzyme activity in daphnids exposed in Falcon tubes or Petri dishes had distinct differences (Figure 2). According to the available literature, there is evidence that suggests the differentiating mechanism of toxicity on a molecular level for AgNPs and Ag$^+$ ions in daphnia [50]. Research on the mechanism of toxicity for AgNPs reports that silver ions cause inhibitory changes to the activity of the Na$^+$, K$^+$-ATPase, which in turn disrupts the normal function of the gills [29,51]. Another suggested mechanism of toxicity for the AgNPs is the disruption of the RNA polymerase via ion binding on specific sites, according to the biotic ligand model for daphnia [52,53], as well as the protein digestion and absorption pathways of the RNA polymerase [54]. Furthermore, Poynton et al. [47] discovered that AgNPs can disrupt proteolysis, which corresponds with our findings showing reduced enzyme activity in both acetic and alkaline phosphatases, as well as peptidases for Petri exposure conditions (Figure 2).

This study posits that the inhibition of the proper phosphorylation of crucial proteins in energy production pathways is balanced out, to some extent, by an elevation in LDH activity (Figure 2), which is likely a compensatory response by daphnids to counteract the energy loss via anaerobic metabolism. The present investigation lends support to this hypothesis as a notable distinction between the two exposed conditions was observed with

regard to enzyme activity for ACP, ALP, and LDH. Our results align with prior research, which has reported an increase in LDH activity in acute exposure of neonates to AgNPs [55]. In contrast to the daphnids exposed in Falcon tubes that exhibited increased phosphatase enzyme activity, daphnids exposed in Petri dishes manifested reduced phosphatase activity and increased LDH activity, also hinting at a distinctive mode of toxicity for the two vessels [50,56].

The exposure of daphnids to silver nano-ink was further evaluated with measurements of their feeding rate [57]. The feeding rate of daphnids is an important endpoint used in daphnid toxicity testing, and, according to the available literature, other crustacean species such as *Gammarus* sp. silver nanoparticles (AgNPs) have even been reported to cause disturbances in their feeding rate [58,59]. Silver nano-ink exposures impacted daphnids exposed in the two tested vessels differently (Figure 4). Dose-dependent effects were not observed for the feeding rate in the Falcon tube as both 0.5 µL/L and 1 µL/L exposure conditions displayed the same impact. The Petri dish exposure condition displayed a dose-depended effect, as the 0.5 µL/L and 1 µL/L exposure conditions had statistically significant differences with their control in terms of observed feeding rate, which further emphasizes the different impact that silver nano-ink had in the two exposure conditions. In contrast to the key enzyme activity endpoint, the control conditions for the feeding endpoint did not display any statistical significant differences. This shows that the two exposure vessels tested do not have any impact on the daphnid feeding rate endpoint before the exposure to pollutants. The findings of Ribeiro et al. [30], which report that AgNPs exposure results in a greater impact on the feeding rate of daphnids compared to the impact of ionic silver exposure, also support this interpretation of results. Our hypothesis for this effect is based on the different dimensions of the two exposure vessels. In OECD media, AgNPs have been reported to rapidly aggregate [60]; therefore, the two exposed groups did not interact with the same form of silver during their exposure periods due to the S:V differences in their respective vessels. There is already evidence in the available literature which suggests that there are differences in silver uptake in daphnia when comparing ionic silver and AgNPs [61,62].

In conclusion, our study highlights the importance of critical thinking in the experimental design of nano-pollutants. However, the knowledge gap in nano-pollutants remains a matter of grave concern. Exploring the importance of different factors in nanotoxicity testing could lead to the development of an experimental design framework for nano-pollutants if the importance of different factors in nanotoxicity testing is explored further. A uniform experimental design for nano-pollutants could greatly benefit research and improve the relevance of the generated results.

4. Materials and Methods

4.1. Culturing Daphnids and Exposures

Cultures of daphnids were maintained in glass beakers in OECD media (final concentrations of 0.29 g of $CaCl_2.2H_2O$/L, 0.123 g of $MgSO_4.7H_2O$/L, 0.065 g of $NaHCO_3$/L, 0.0058 g of KCl/L, and 2 µg of Na_2SeO_3/L, pH 7.7) under a 16 h:8 h light–dark photoperiod at 20 °C. All chemicals used in this study were of the highest purity >99.9% and quality. KCl, Na_2SeO_3, latex beads, carboxylate-modified polystyrene, fluorescent red, bovine serum albumin, brilliant blue G, *p*-nitrophenyl butyrate, 2-nitrophenyl-B-D-galactopyranoside, 1-chloro-2,4-dinitrobenzene, L-glutathione reduced, sodium phosphate dibasic were purchased from Sigma-Aldrich. 4,4'-dithiodipyridine, $CaCl_2·2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$, HCl, *p*-nitrophenyl phosphate, boric acid, ammonium acetate and NaOH were purchased from Fisher Scientific. The media were renewed every five days and the cultures were fed daily with *Chlamydomonas rheinharti* suspension and an organic seaweed extract (*Ascophylum nodosum*) only upon media renewal. For silver nanoparticle ink exposures, the neonates (<24 h) were collected from the third brood of their mothers and cultured until the age of four days old. Animals were exposed for 24 h in Falcon tubes and Petri dishes (Figure 5). Fifteen daphnids were used per replicate of each vessel in 50 or 100 mL of volume and

full toxicity curves were obtained with a minimum of three experiments conducted to reduce differences between the different batches of animals in mortality (Figure 1). All plots were calculated using the four parameter logistic (4PL) model, following the equation Span = Top − Bottom and Y = Bottom + (Top-Bottom)/(1 + 10^((LogIC50-X) × HillSlope)) using GraphPad software. The top and bottom parameters were commonly fixed to 100 and 0, accordingly.
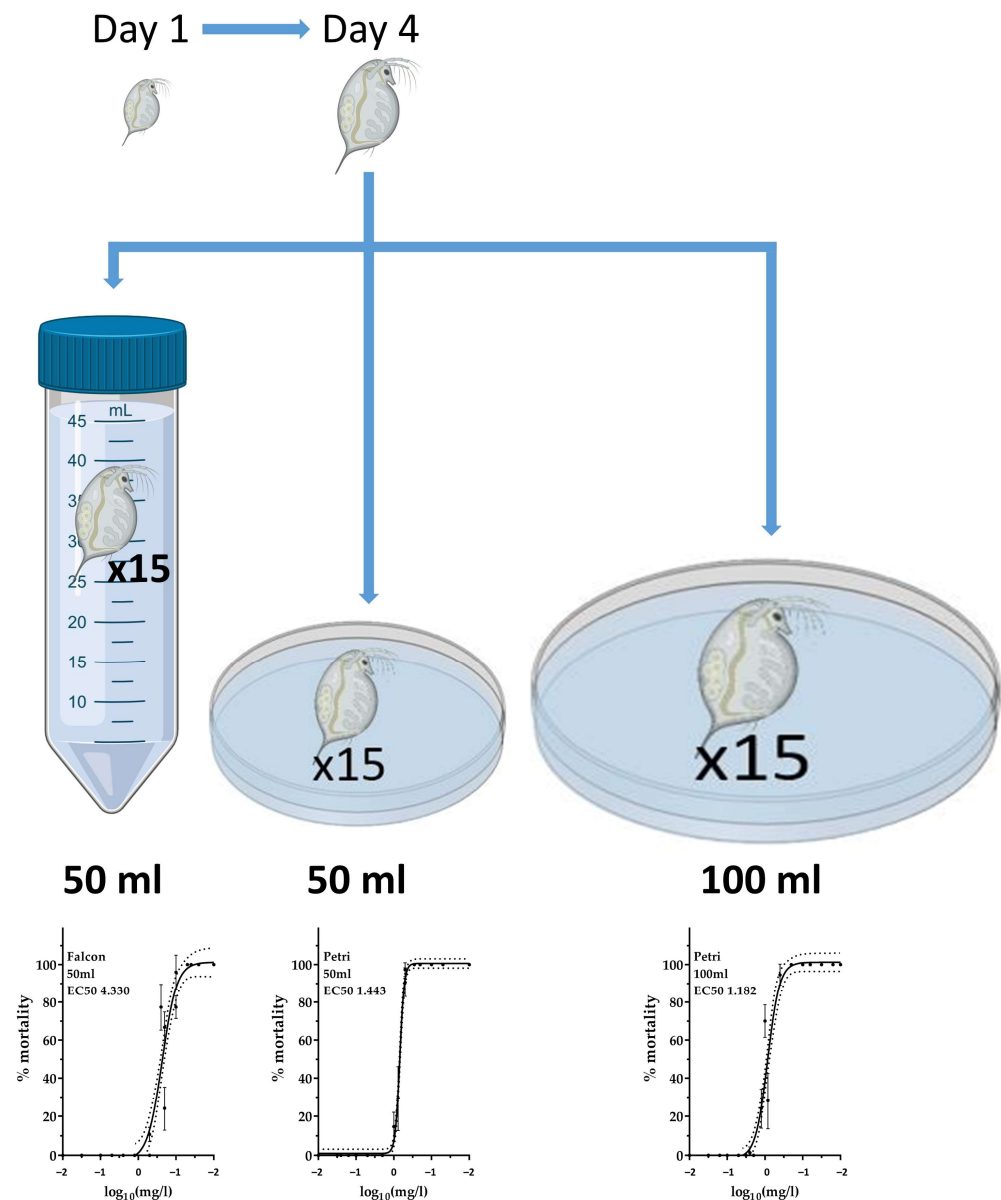
Figure 5. Experimental design for exposures of daphnids to silver nano-ink in Falcon tubes and Petri dishes.

4.2. Exposure of Daphnids to Silver Nanoink and Markers of Physiology

Daphnids (four days old) were exposed to 0.5 µL/L and 1 µL/L of silver nanoparticle ink for 24 h. Thirty daphnids were pooled together and homogenized in a 0.5 mL buffer using a pestle homogenizer. The homogenate was centrifuged and the clear supernatant was collected and assessed for protein and enzyme activity. The protein was quantified using a sensitive Bradford method [63] to normalize the enzyme activity. The activity of phosphatases was assayed in 100 mM citric acid (pH 4.5) (for ACP) or 100 mM boric acid (pH 9.8) (for ALP) using the substrate *p*-nitrophenyl phosphate. The reaction was alkalined and the absorbance of produced *p*-nitrophenol was measured at 405 nm and

converted to units per protein. Similarly, the activities of BGAL and LIP were quantified via the generation of nitrophenol from the catalysis of *o*-nitrophenyl-β-galactoside or *p*-nitrophenyl butyrate, respectively, in a phosphate buffer (pH 7.2). The activity of the peptidase (specifically γ-glutamyl transferase) was quantified using L-leucine-4-nitroanilide as a substrate. Then, 200 μL of the sample, appropriately diluted in a 50 mM phosphate buffer (pH 7.2), was mixed with 50 μL of the 8 mM substrate in 100% DMSO, and the release of the product (*p*-nitroanilide) was measured by continuous kinetics at 418 nm [64]. GST activity was measured via the formation of a complex between reduced glutathione with 1-chloro-2,4-dinitrobenzene at 340 nm [65]. The activity of LDH was quantified by monitoring the decrease in absorbance at 340 nm caused by the oxidation of NADH. The substrate used was a 1:1 mixture of 40 mM pyruvate and 0.5 mM NADH. In addition, 200 μL of the sample, appropriately diluted in the 50 mM phosphate buffer (pH 7.2), was mixed with 50 μL of the substrate. The resulting reaction was measured via continuous kinetics at 340 nm.

4.3. Feeding Assay

Carboxy microplastics (L3030, Sigma Aldrich, St. Louis, MO, USA) were used in this study to assess the ingestion (feeding) rate of daphnids. These microplastics were tested initially using 1-day-old neonates as they are more sensitive, and no toxicity was recorded for up to 10 h of exposure to microplastics. The concentrations used for this assessment ranged up to 52 mg/L; however, for the feeding assay, a concentration of 13 mg/L was used. The fluorescence from microparticles was measured as Ex/Em 560/590 nm using a TECAN plate reader (Männedorf, Switzerland). The ingested microparticles were measured twice, first as fluorescence in the media during the feeding assay, and the fluorescence difference in the media from the initial time of incubation was converted to the amount of ingested microplastic using a standard curve and expressed per animal. Then, the feeding rate of daphnids was measured after 30 min from the ingested carboxy microplastics following the homogenization of animals and the extraction of microparticles in 0.5 mL of ddH$_2$O. The ingestion of microparticles was also confirmed with fluorescence microscopy using the TRITC filter (Nikon Eclipse TS100). A separate pool of animals was incubated in the absence of microplastic as a negative control for any animal background fluorescence.

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