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Bioaccumulation and Ecotoxicity of Silver Nanoparticles in Simple Food Chain Algae-Microcrustaceans in the Presence of Natural Organic Matter

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Abstract: The rapid growth of nanotechnology has resulted in widespread use of nanoparticles (NP) in various commercial products. Consequently, these NP may enter aquatic environments, where they can interact with natural organic matter (NOM) such as humic substances and extracellular polymeric substances (EPS). The present work aims to evaluate the toxicity of manufactured coated silver nanoparticles (AgNP) in the presence of NOM on the microalgae *Raphidocelis subcapitata* and the microcrustacean *Daphnia similis*, both individually and within a simple food chain. By investigating the influence of NOM on the bioavailability and toxicity of AgNP, this research seeks to understand the potential environmental risks associated with these nanomaterials. The test-organisms, individually and simple food chain, were exposed to AgNP manufactured coated (citrate and poly(ethylene glycol), respectively Cit and PEG) and ionic silver solution ($AgNO_3$) in the absence and presence of NOM, represented by humic substances (HS) and natural EPS (excreted by algae). Algae growth inhibition was assessed, and the toxic effect of silver ions remained unchanged in the presence of NOM. However, the toxicity of the highest of AgCit was reduced by HS. NOM enhanced silver bioaccumulation in algae, likely due to surface binding of HS-Ag complexes. HS exposure to AgCit decreased silver bioaccumulation, correlating with reduced toxicity. Microcrustaceans exhibited greater sensitivity to silver toxicity compared to algae. The toxic effects on Daphnia similis were evaluated through mortality (EC_{50}) and reproduction (number of neonates). The toxicity ranking was $AgNO_3$ > AgCit > AgPeg, indicating higher toxicity for ionic silver. Daphnia reproduction was observed at environmentally permitted concentrations. Different toxic effects of AgNP were observed between neonate and adult microcrustaceans, suggesting age-dependent sensitivity. These findings highlight the need for bioassays considering the influence of NOM and capping agents to better understand the environmental impact of nanomaterials.

Keywords: environmental chemistry; nanoparticles; ecotoxicology; humic substances; water contamination

1. Introduction

The current growth of nanoscience and nanotechnology, coupled with the widespread use of manufactured nanoparticles (NP) in commercial products, one dimension in the nanoscale (1–100 nm), could lead to an increased presence of these materials in the biosphere [1]. As nanoparticles (NP) represent an emerging contaminant class with significant potential for environmental release, it is crucial to assess their potential impact, particularly within aquatic ecosystems [2]. Exposure to nanomaterials (NMs) can occur at various stages, from production, handling, processing, use, and disposal [3].

Recently, due to the need for an effective agent against SARS-CoV-2 during the pandemic, extensive research has attested the antiviral effect of AgNP as a highly potent microbicide against SARS-CoV-2 [4,5]. However, these NMs should be used with caution due to their cytotoxic effects and their potential to disrupt environmental ecosystems. Furthermore, manufactured NP can interact with abiotic molecules in the environment, producing transformed NP with distinct new properties. However, due to contradictory findings in the literature, the environmental implications of these interactions remain uncertain. The toxicity of nanoparticles has been widely studied in aquatic organisms, ranging from decomposers (bacteria), primary producers (algae), and secondary consumers (micro-invertebrates and fish).

Algae are typically microscopic organisms that subsist on inorganic nutrients and produce organic matter from carbon dioxide through the process of photosynthesis [6]. They can exist as single cells, filaments, sheets, or colonies. Commonly known as green algae, Chlorophyta are responsible for a large portion of primary productivity in freshwater environments. Algae play a crucial role in aquatic ecosystems by producing biomass through photosynthesis, which fixes carbon dioxide and inorganic carbon from dissolved carbonate species into organic matter, thereby providing the foundation of the food chain for other organisms. Moderate levels of algal biomass production are essential for maintaining healthy aquatic ecosystems, supporting biodiversity, and ensuring the overall balance of the ecosystem [7]. *Raphidocelis subcapitata* is a single-celled alga known for its rapid growth, making it easy to culture and a representative organism of freshwater environments [8]. Bioaccumulation, defined as the accumulation of substances through both bioconcentration (uptake via body surfaces) and biomagnification (assimilation through dietary intake), is primarily attributed to bioconcentration, as supported by existing literature [9,10]. Daphnia sp. is a species of small freshwater crustaceans (order Cladocera) classified as zooplankton [11]. These organisms occupy a crucial position in aquatic food webs, consuming algae (primary producers) and serving as a food source for a variety of higher trophic level organisms, including fish and vertebrates [12]. Trophic transfer studies serve as a valuable tool for evaluating environmental risks by enabling the distinction of the relative importance of various exposure pathways [13].

Humic substances, a complex and heterogeneous fraction of dissolved organic matter (DOM) in freshwater environments, play a crucial role in various ecological processes, including the reduction of metal toxicity to aquatic organisms. Recent studies suggest that humic substances can also directly influence stability, and consequently, the toxicity of nanoparticles [14,15]. Understanding the interactions between humic substances and nanoparticles, including the transformations that occur, is essential for assessing the environmental risks of these emerging contaminants and developing effective mitigation strategies.

Ecotoxicological assays are conducted using standardized methods to assess toxicity and establish water quality criteria. While lethality is the most common effect observed in toxicity tests, contaminant concentrations in natural environments are rarely high enough to cause direct mortality [12]. It is well-established that free silver ions (Ag^+) are highly toxic to a wide range of organisms, including bacteria [16]. In the case of silver nanoparticles, dissolution is the primary mechanism of toxicity, releasing toxic silver ions [9,17,18].

To our knowledge, there are no studies in the literature that have evaluated the behavior of AgNP in the presence of NOM and exposure to algae and microcrustaceans of simple food chains. Therefore, this study elucidates the significant role of NOM in the toxicity of NP within aquatic systems, further addressing their bioaccumulation along the food chain. For this purpose, we evaluated the toxic effects of coated silver nanoparticles (AgNP) manufactured on the microalgae *Raphidocelis subcapitata* and the microcrustacean *Daphnia similis* in the presence and absence of humic substances (HS). In addition, we investigated the impact of different exposure routes (aquatic and dietary) on the toxicity of AgNP using juvenile and adult *D. similis* to better understand their ecological risks in aquatic environments.

2. Materials and Methods

2.1. NOM Extraction

Humic substances (HS) were extracted using a standard chromatographic method with a DAX-8 resin column (Superlite™, Sigma Aldrich, St. Louis, MO, USA), a recommended technique used for isolating aquatic humic substances (AHS) International Humic Substances Society (IHSS), with adaptations [19–21]. The schematic procedure for HS extraction is depicted in Figure 1. HS was extracted from water samples collected from the Sorocabinha River, a hydrographic region in Iguape, Sao Paulo, Brazil, (24°41′59″ S, 47°33′05″ W) known for its extensive mangrove areas and high natural organic matter (NOM) (① in Figure 1). In situ measurements of electrical conductivity, pH, and dissolved oxygen were conducted. Additionally, laboratory analysis of biochemical oxygen demand (BOD5) was performed after a 5-day incubation period at 20 °C. Water samples from the Sorocabinha River were acidified to pH < 2 with HCl (1 mol·L^{-1}) to facilitate HS retention on the resin (② in Figure 1). The purified resin was then packed into columns, acidified with 0.1 mol·L^{-1} HCl to optimize AHS fixation, and used to retain AHS as water slowly percolated through (③ in Figure 1). Finally, the retained AHS were eluted with 0.1 mol·L^{-1} NaOH (Dinamica, Brazil) (④ in Figure 1), acidified to pH 5.8 (matching the original water sample pH using HCl 0.1 mol·L^{-1}, Table S1) (⑤ in Figure 1), and stored for subsequent experiments. For the toxicity tests using HS, total organic carbon (TOC) was quantified by a Total Organic Carbon Analyzer (multi N/C 3100, Analytik Jena, Jena, Germany), with concentrations expressed in mg of organic carbon per liter (mg·C·L^{-1}) through the bioassays.

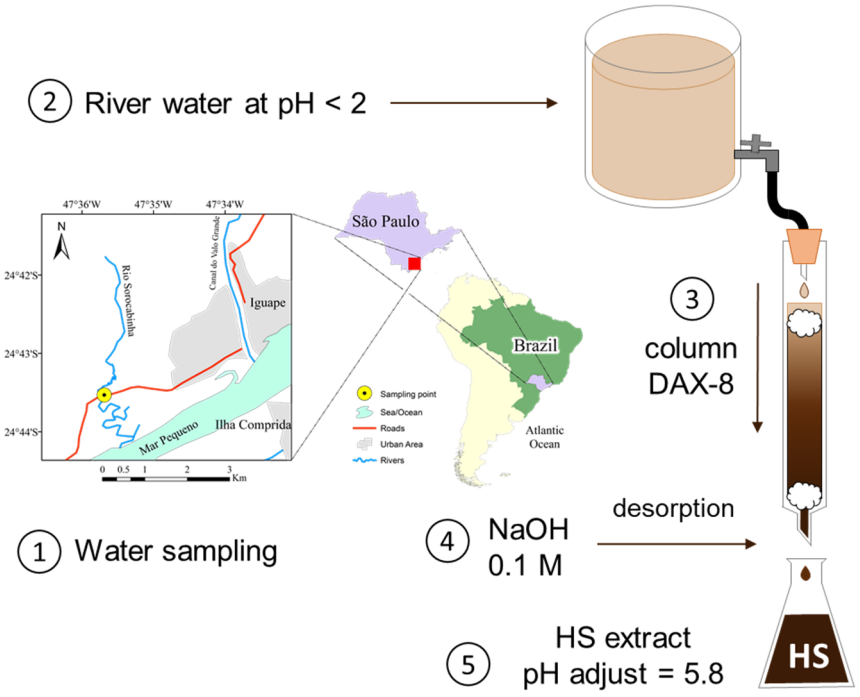

Figure 1. Schematic HS extraction from Sorocabinha River. ①. Map of water sampling region; ②. Water sampled acidified at low pH. ③. Extraction process apparatus using DAX-8 column; ④. HS obtention by NaOH 0.1 M; ⑤. HS final extract (pH 5.8).

2.2. Raphidocelis subcapitata Bioassays

Bioassays with *Raphidocelis subcapitata* were conducted according to standard protocols outlined by the United States Environmental Protection Agency (USEPA), Organisation for Economic Co-operation and Development (OECD), and Associação Brasileira de Normas Técnicas (ABNT) [22–24]. *R. subcapitata* cultures were maintained according to (ABNT, 2018). These organisms were used for both bioaccumulation tests and as food in *D. similis* cultures. The L.C. Oligo medium, composed of seven nutrient solutions (available in Table S2), was used to provide *R. subcapitata* growth. A sensitivity test was conducted to assess the health of algae cultures. Details of the sensitivity test, including solution preparation (Table S3) and spectrophotometric cell counting (Figures S1 and S2), are provided in the Supplementary Information.

To initiate the bioassays, known volumes of algal inoculum were added to the test solutions to achieve an initial cell density of 10^4 to 10^5 cells·mL^{-1}. Aliquots were collected at the beginning of the exposure period to accurately determine the initial cell concentration. The samples were incubated under constant light and agitation for 72 h, with flasks being randomly re-positioned every 24 h. After the experiment, the test solutions were fixed with Lugol's solution (80 mL per 2 mL of sample) and cell counts were performed using a Fuchs-Rosenthal chamber (Supplementary Information). The obtained cell counts were used to calculate EC50 values through statistical analysis using the ICpin software.

To assess the potential toxicity of the extracted HS, toxicity tests were conducted using only the Oligo medium and HS at a concentration of 10 mg·C·L^{-1}. The experimental procedures followed the guidelines outlined for algae protocols [24]. Additionally, toxicity tests were performed using the matrix solutions employed during the synthesis of the two nanoparticles under investigation.

2.2.1. $AgNO_3$ Bioassay

A standard silver nitrate solution was prepared by dissolving 0.16 g of dried $AgNO_3$ powder in 1 L of deionized water [25]. This solution contained 0.1 mg·Ag·mL^{-1}.

To determine the EC50 value for silver ions, a preliminary bioassay was conducted with *R. subcapitata* using a concentration range of 1 to 100 µg·Ag^+·L^{-1}. Subsequently, a second bioassay was performed using 2 specific concentrations: 1 and 100 µg·Ag^+·L^{-1} in the absence and presence of HS (1 and 10 mg·C·L^{-1}). All test solutions, including a control containing only the nutrient medium, were prepared in triplicate. The samples were incubated in a shaking incubator at 25 ± 2 °C and 175 rpm under constant light for 96 h. The position of the test vessels was randomized every 24 h.

2.2.2. Silver Nanoparticles Bioassays

Raphidocelis subcapitata bioassays were conducted using two concentrations of silver ions (Ag^+): 1 and 100 µg·L^{-1}. These assays were performed with $AgNO_3$, AgCit, and AgPeg in the absence and presence of natural organic matter (HS1 and HS2) at concentrations of 1 and 10 mg·C·L^{-1}. The experimental procedures followed the guidelines outlined [22,23]. To assess potential acute toxicity, algal growth in the test and control groups was compared using Fisher's Exact Test with a significance level of $p < 0.05$, as analyzed by Toxstat 3.5 (West and Gulley, 1996).

2.2.3. Accumulation Experiments

Bioaccumulation tests were conducted concurrently with toxicity assays (to visualize the detailed experimental procedure, please refer to Figure S3). At specific time points (0, 72, and 96 h), 40 mL aliquots of each test solution were filtered through 0.45 µm cellulose acetate filters (Sartorius) using a vacuum pump. To minimize contamination, filters were pre-treated with 5% HNO_3 and subsequently washed with ultrapure water. After filtering 40 mL of the test solution, 5 mL of 0.1 mol·L^{-1} EDTA was added to remove any adsorbed metals from the cell walls. Subsequently, 0.3 mL of concentrated HNO_3 was added to digest the bioaccumulated metals. The unfiltered test solution was partially acidified with HNO_3 to provide a total metal concentration reference. To determine the bioaccumulated metals, the filters were digested in 0.3 mL HNO_3 at 85 °C for 12 h. The resulting solutions were analyzed to quantify metal uptake by the algae by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

2.3. Daphnia Similis Bioassay

Physicochemical parameters, including pH, dissolved oxygen, conductivity, and hardness, were measured at the end of each experiment according to standard methods [26]. Data are available in the Supplementary Information.

2.3.1. Acute Toxicity

Acute toxicity tests were conducted following OECD Guideline [27,28] and incorporating specific conditions for *Daphnia similis* culture outlined in ABNT NBR 12713 [29]. For *Daphnia* medium exposure solution (Table S4), soft reconstituted water (hardness) was adopted from OECD 202 [27]. A range of $AgNO_3$ concentration (1, 2, 5, 10, 25, 50, 100 µg·Ag^+·L^{-1}) was tested to determine the EC50 value for silver ions.

The test assesses the immobility effect of the organism exposed to concentrations of a reference substance and can be expressed by EC50 (for *Daphnia*). Neonates of *D. similis* (6–24 h old) were exposed to each test

concentration (1 and 100 μg·Ag^{+}·L^{-1}) for 48 h without food and water renewal. EC50 values were calculated using the Trimmed Spearman-Karber method [30]. A schematic representation of the experimental setup is provided in Figure S4.

2.3.2. Long-Term Exposure Bioassays

Long-term exposure bioassays were conducted according to OECD Guideline 211 [28] for *D. magna* reproduction. *D. similis* were fed yeast and Tetramin, with water renewal every 2–3 days. Reproduction was monitored for 21 days.

2.3.3. Waterborne and Dietborne Comparative Assays

For algae-microcrustacean experiments, the algae were exposed to Ag^{+} and/or AgNP concentrations during 96 h. Neonates (age between 6–24 h) and adults (day 7) were exposed during 48 h at two concentrations (1 and 100 μg·L^{-1}) of $AgNO_3$, AgCit and AgPeg nanoparticles in both presence and absence of HS (1 mg·L^{-1} as HS1 and 10 mg·L^{-1}, as HS2). Control solution without Ag, $AgNO_3$, AgCit and AgPeg, only humic substances 10 mg·L^{-1} (HS2), was also performed.

2.3.4. Statistical Analyses

For acute response bioassay using *Daphnia similis* 48 h test, the EC50 response for Ag^{+}, AgCit and AgPeg were calculated using Trimmed Spearman Karber (TSK) software [30].

For long-term exposure, the toxic response was assessed using Fisher's Exact Test performed by Toxstat 3.5 [31] in order to identify eventual acute toxic effects. Treatments exhibiting significant differences ($p < 0.05$) were excluded from further assessment of reproduction response. The reproduction data (offspring) from the remaining treatments were then tested for normality (using Chi-square and Shapiro-Wilk's tests) and homogeneity (using Hartley's and Bartlett's tests). Subsequently, the data were subjected to one-way analysis of variance (ANOVA) with a Bonferroni post-hoc t-test for comparisons between control means and treatments. A significant level of 5% ($p < 0.05$) was applied to all analyses. Toxstat 3.5 was also used to compare the immobility assessment of control and test exposition in water and dietborne exposition using Fisher's Exact Test significance level ($p < 0.05$).

3. Results and Discussion

3.1. $AgNO_3$ Bioassays

The EC50 results are presented in Figure 2. The EC50 = 17.83 ± 1.18 μg·L^{-1} in terms of Ag^{+}. Similar values can be found in the literature (EC50 = 33.77 μg·L^{-1}) [32]. $AgNO_3$ toxicity results in the presence of HS at a concentration of 1 μg·L^{-1} can be seen in Figure 2. When comparing the EC50 values obtained for silver ions (Figure 2a) with the lowest concentration evaluated (1 μg·L^{-1}), no toxic effect on *R. subcapitata* was observed, either in the presence or absence of organic matter (Figure 2b). Although there was no significant difference from the control, a considerable increase in cell number can be observed at the lowest HS1 concentration (1 mg·C·L^{-1}) compared to the higher HS2 concentration (10 mg·C·L^{-1}). For the highest Ag^{+} concentration, as expected by the toxicity assay for EC50 value, all treatments using 100 μg·L^{-1} (Figure 2c) exhibited high toxicity, indicating that the presence of HS did not mitigate this toxicity.

Literature reveals research work has tested different concentrations of organic matter onto microalgae, yielding similar results to those found here [33]. For instance, the authors tested different concentrations of humic acid (HA) to treat the microalgae *Scenedesmus capricornutus*. As for the results, at HA concentrations lower than 2.0 mg·C·L^{-1} the growth of *S. capricornutus* was slightly promoted, while concentrations above 2.0 mg·C·L^{-1} inhibited growth. The same study also reported an increase in polysaccharide content at low HA concentrations (<2.0 mg·C·L^{-1}), which decreased as the HA concentration increased. More recent studies have demonstrated that higher doses of H2S from various sources inhibited microalgal growth. This dual response is typical of hormesis, a dose-response phenomenon characterized by low-dose stimulation and high-dose inhibition. Hormetic effects are commonly represented graphically as an inverted U-shaped and a J-shaped dose-response, depending on the endpoint evaluated [15].

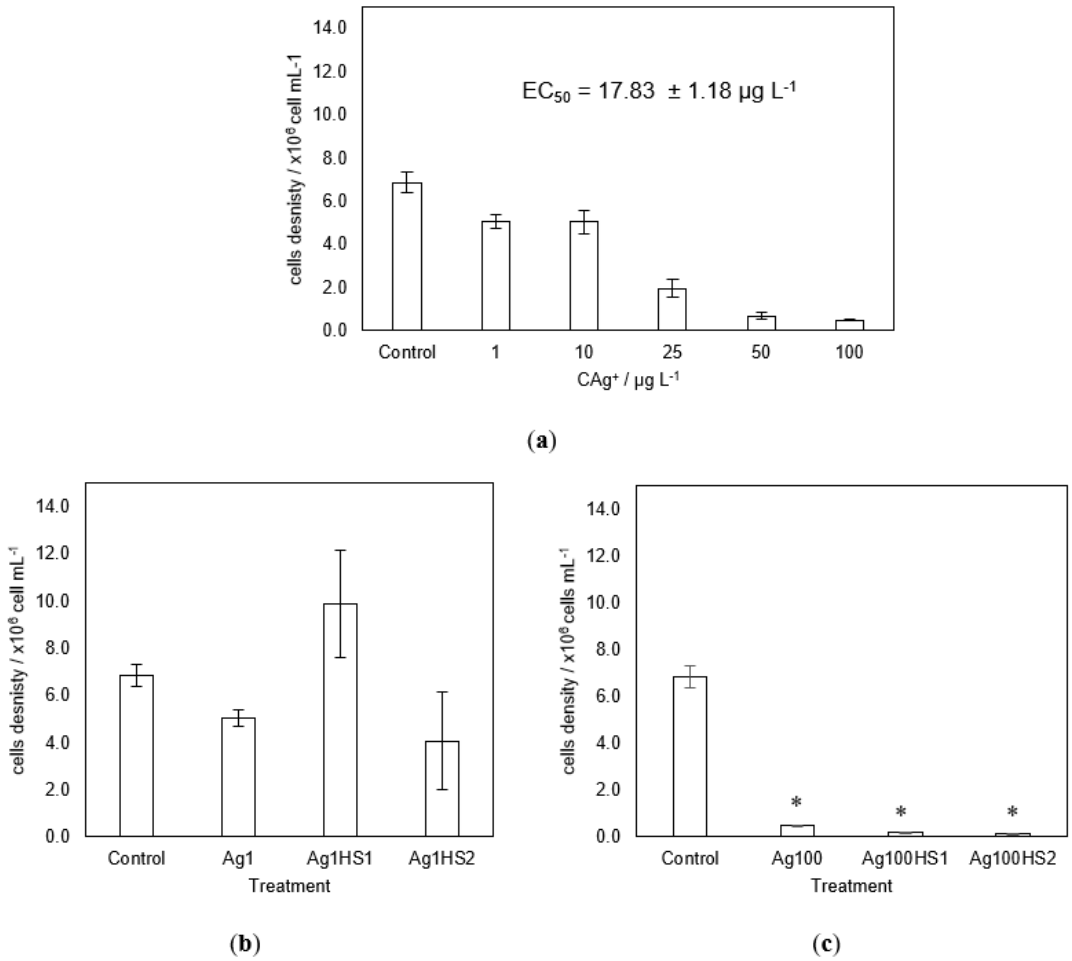

Figure 2. Toxic effect of green algae *R. subcapitata* exposed to (**a**) $AgNO_3$ (**b**) 1 and (**c**) 100 µg·L^{-1} $AgNO_3$ in the presence of HS1 = 1 mg·L^{-1} HS; HS2 = 10 mg·L^{-1} HS. Mean values ± SD (N = 3). The significant differences are indicated by * for $p \leq 0.05$

3.2. AgCit Bioassays

Figure 3 indicates results from AgNP bioassays. Mean values of cell density were obtained from triplicate each bioassay (N = 3). The significant differences are indicated by $p < 0.05$. Figure 3a,b illustrates the toxicity outcomes of citrate-coated nanoparticles across two concentrations (1 and 100 µg·L^{-1}). For the lower nanoparticle concentration, a statistically significant difference relative to the control was detected exclusively in the presence of humic substances at their maximum concentration. Notably, this deviation resulted in stimulated cell proliferation rather than inhibition of algal growth. In contrast, for the higher concentration of citrate-coated nanoparticles, no toxic effects were evident, either in the absence or presence of HS at any tested organic matter concentration (Figure 3b). The absence of toxicity observed in the algae during our experiments is likely attributed to the increased cell number of *R subcapitata* with rising humic acid concentrations within the tested range, although this increase was not statistically significant [34].

Our toxicity results indicate a non-toxic effect in the presence of aquatic humic substances. This observation supports the hypothesis from the literature that reduced AgNP dissolution, often attributed to the presence of humic substances [18]. One such hypothesis may be related to the stabilization that organic matter can provide to the coated NM. According to the literature, natural entities bind to the surface of NP, forming a coating around them, affecting their surface reactivity and interactions with cells and organisms [35,36]. NOM can promote electrostatic or steric stabilization of NP or result in flocculation [37]. It is worth remembering that the presence of long-chain polysaccharides secreted by microorganisms, the Exopolysaccharides (EPS), in the system may influence the stabilization of NMs, which is why there is no toxicity effect even in the absence of AHS.

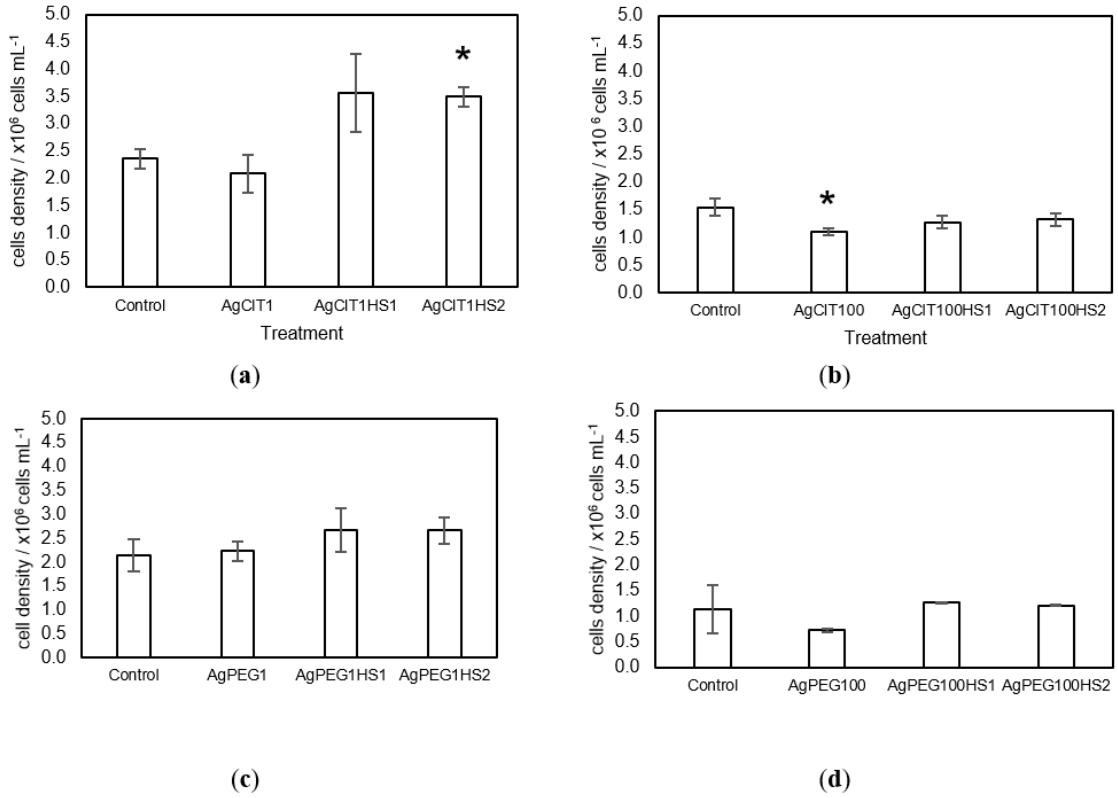

Figure 3. Toxic effect of *R. subcapitata* green algae in the presence of AgCit (**a**) 1 μg·L^{-1} and (**b**) 100 μg·L^{-1} and AgPeg (**c**) 1 μg·L^{-1} and (**d**) 100 μg·L^{-1}. Mean values ± SD (N = 3). HS1 = 1 mg·L^{-1} HS; HS2 = 10 mg·L^{-1} HS. The significant differences are indicated by * for $p \leq 0.05$.

3.3. AgPeg Bioassays

The toxicity results for AgPeg are presented in Figure 3c,d. The results reveal no significant difference across all exposure conditions, including the presence and absence of HS and various AgPeg concentrations (1 and 100 μg·L^{-1}) tested. The high standard deviation of the control data merits attention, as it may have led to a false negative assessment of the toxic effect. Furthermore, no direct relationship was observed between the reduction of toxicity and nanoparticle dissolution when comparing our toxicity data with dissolution behavior [18].

AgCit EC50 values under *R. subcapitata* microalgae after 96 h were equivalent to 32.40 μg·L^{-1}. Significant differences in the growth rate of the control sample exposed to 100 μg·L^{-1}. The stability of AgNP when influenced by humic substances is understood to depend on both the type of humic substance and the nanoparticle's surface coating [38]. Research indicates that the aggregation rate of AgNP, when exposed to humic and fulvic acids, varies with the chemical composition of these organic materials [39]. Furthermore, interactions between AgNP and humic and fulvic acids have been observed to induce alterations in nanoparticle charge and stabilization. For instance, the proton release from sodium citrate-coated AgNPs has been reported to decrease upon exposure to acidic solutions [39]. Beyond silver, studies on other nanoparticles, such as $nCeO_2$, have also shown that varying concentrations of humic substances can mitigate their toxicity to microalgae like *R. subcapitata* and bacteria such as *C. sphaericus* [40].

The interaction between humic substances and silver nanoparticle coatings plays a large role in these results, since it is this nanoparticle coating that directly interacts with the natural organic matter present in the solution. Studies have shown that the use of humic substances as reducing and stabilizing agents during the preparation of nanoparticles is attractive because of their ability to improve the colloidal stability of the solution, as well as reducing the use of toxic reagents during this procedure.

The antimicrobial and fungicidal function of these AgNP would be due to the release of these Ag^+ ions into the medium. Other possible environmental impacts of these nanoparticles in the medium are the large aggregation capacity of the particles, which influences the sedimentation rate and the mobility of these nanomaterials in the environment [41,42]. Interactions between nanoparticles and humic substances can prevent problems from extending to this extent and help to counteract the adverse effects of silver nanoparticles on the medium.

3.4. Bioaccumulation of AgNP

The bioaccumulation results are presented in Figure 4. The *R. subcapitata* bioassay results (Figure 4a), using silver nitrate in the presence and absence of organic matter, indicate that single silver ion bioaccumulation was lower compared to the ions exposed to HS2, as depicted by the red dashed line in Figure 4a. Additionally, a proportional increase in Ag accumulation over time can be observed for both treatments ($AgNO_3$ and $AgNO_3$HS2). Bioaccumulation in *R. subcapitata* exposed to AgCit was lower than when exposed to $AgNO_3$. Quantitatively, the mass accumulation of Ag was substantially greater for ionic Ag ($AgNO_3$) exposure than for Ag from AgNP. Nevertheless, the temporal increase in Ag accumulation displayed a pattern similar to that observed with $AgNO_3$. Figure 4b further indicates that the presence of HS mitigated bioaccumulation, a reduction that was dependent on HS concentration but independent of exposure duration.

Studies have shown that *R. subcapitata* accumulates more silver as the concentration of silver thiosulfate complexes in the medium increases [43]. This phenomenon is consistent with the understanding that the algal cell wall's pore diameter (5 to 20 nm) allows only smaller nanoparticles to be internalized [44]. Given their free nature, silver ions may induce toxicity even when humic substances are present. Furthermore, nanoparticle agglomeration warrants consideration in bioaccumulation and toxicity analyses, as aggregated nanoparticles are known to adsorb onto the outer surface of algal cells [45]. Consequently, the aggregation rate may impair cell division, resulting in a reduced growth rate and compromised photosynthesis. Such findings are consistent with observations from studies on silver ion toxicity in the presence of humic substances, which have revealed similar effects on growth [46,47]. These studies demonstrated that despite increased silver uptake in the presence of humic substances, algal concentration was not diminished. Furthermore, the results suggested that the observed increase in silver uptake when organic matter is present is due to surface-bound silver, implying it is not truly internalized by the algae.

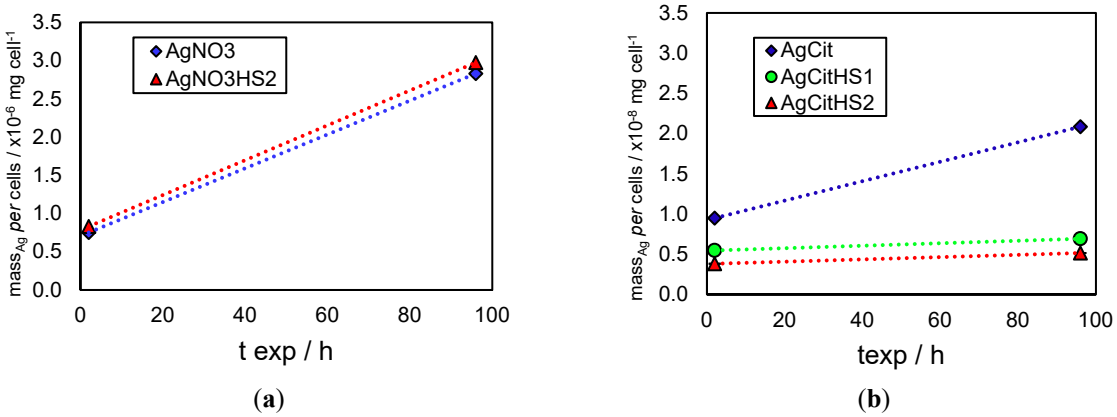

Figure 4. Bioaccumulation results of (**a**) $AgNO_3$ and (**b**) AgCit in the presence and absence of aquatic humic substances. HS1 = 1 mg·L^{-1} HS; HS2 = 10 mg·L^{-1} HS after bioassays of green algae *R. subcapitata*.

Considering the toxic response of *Raphidocelis subcapitata* and comparing the bioaccumulation of citrate-capped nanosilver with that of ionic silver, it is reasonable to conclude that the accumulated silver was significantly higher for the ionic form, which also induced greater toxicity compared to the citrate-capped nanosilver response. The uptake of silver nanoparticles (1.0×10^{-8} mg Ag) was significantly lower than that of $AgNO_3$ (1.0×10^{-6} mg Ag), by two orders of magnitude. For AgPeg, a clear behavior was not evidenced, as no bioaccumulation was measured, with all measurements falling below the quantification limit. The literature indicates that nanoparticle toxicity can be governed by physical or mechanical effects, with the nanoparticles disrupting physiological patterns due to a barrier formed on the algal surface [48]. It has been observed in the literature that organic matter can modulate the bioavailability of various substances to organisms via complexation. Specifically, for algae, organic matter can adsorb onto the cell's exterior, physically obstructing surface pathways essential for cellular uptake. Analogously, nanomaterials might exert a similar effect by interacting with the algal surface, thus hindering vital cellular functions or the internalization of essential elements [49,50]. Furthermore, studies on the dissolution of AgPeg propose an alternative explanation for observed effects [18]. According to their findings, almost 40% of dissolution occurs, releasing enough silver ions to induce toxic effects (EC50 for silver ions = 17.83 µg·L^{-1}).

3.4. Daphnia Similis Bioassays

The data of the sensitivity test expressed in EC_{50} obtained using NaCl as a reference toxicant is available in Figure S5.

3.4.1. Acute Bioassays

The EC50 graph for *D. similis* can be seen in Figure 5. For $AgNO_3$ the value of EC50 = 6.52 $\mu g \cdot L^{-1}$ shows a higher sensitivity of microcrustacean compared to the microalgae EC50 results obtained for *R. subcapitata*. Studies reported in the literature have shown that daphnids were sensitive to $AgNO_3$ exposure obtaining a 48-h LC50 of 2.51 $\mu g \cdot L^{-1}$ and chronic toxicity after 21 days of exposure [51].

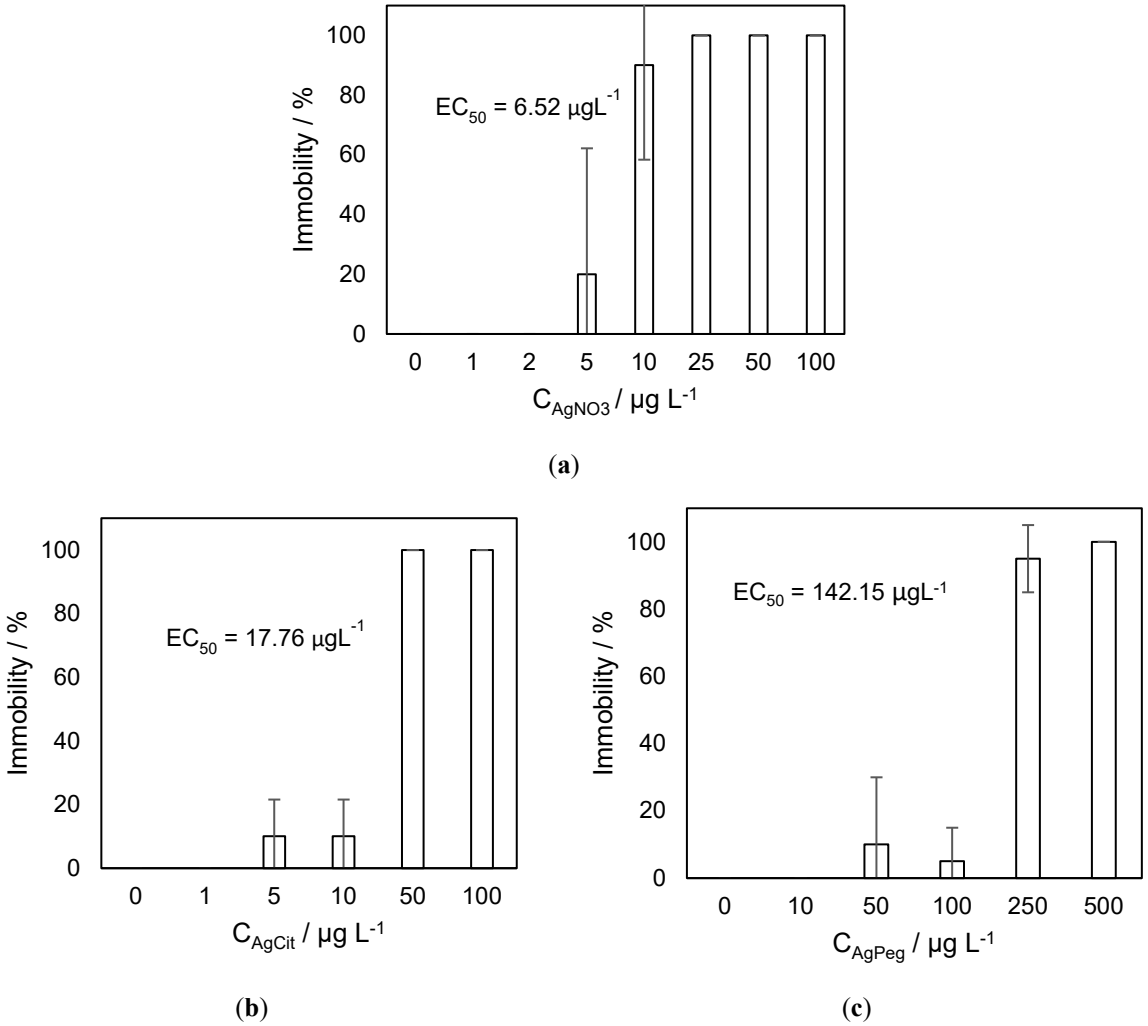

Figure 5. EC_{50} to D. similis results of (**a**) $AgNO_3$ and (**b**) AgCit and (**c**) AgPeg (100 $\mu g \cdot L^{-1}$).

Filtering organisms, by nature of their feeding strategy, often exhibit elevated internalization flows, which can result in rapid exposure and subsequent toxicity [52]. While some studies report that high concentrations of natural organic matter (NOM) mitigate AgNP toxicity to *Ceriodaphnia dubia*, other findings indicate increased toxicity to *Daphnia*-like organisms at 50 $mg \cdot L^{-1}$ AgNP and pH 7, even in the presence of substantial NOM [53]. Integrating studies on AgNP toxicity within the trophic chain with data on AgNP dissolution rates could advance our understanding of internalization and toxicity mechanisms in these microcrustaceans.

3.4.2. Long-Term Exposure Bioassay

The results of long-term exposure are shown in Figure 6. For the highest concentration tested, the acute test, analyzed by Fisher's Exact Test, revealed significant differences compared to the control across all treatments. In some instances, reproduction was still observed, despite high mortality by the end of the test, as it is possible to

observe in the Supplementary Information section. Specifically, an acute effect was noted for AgCit and AgPegHS2 in the 1 $\mu g \cdot L^{-1}$ Ag^{+} test. No chronic effects were observed in other treatments, based on reproduction data.

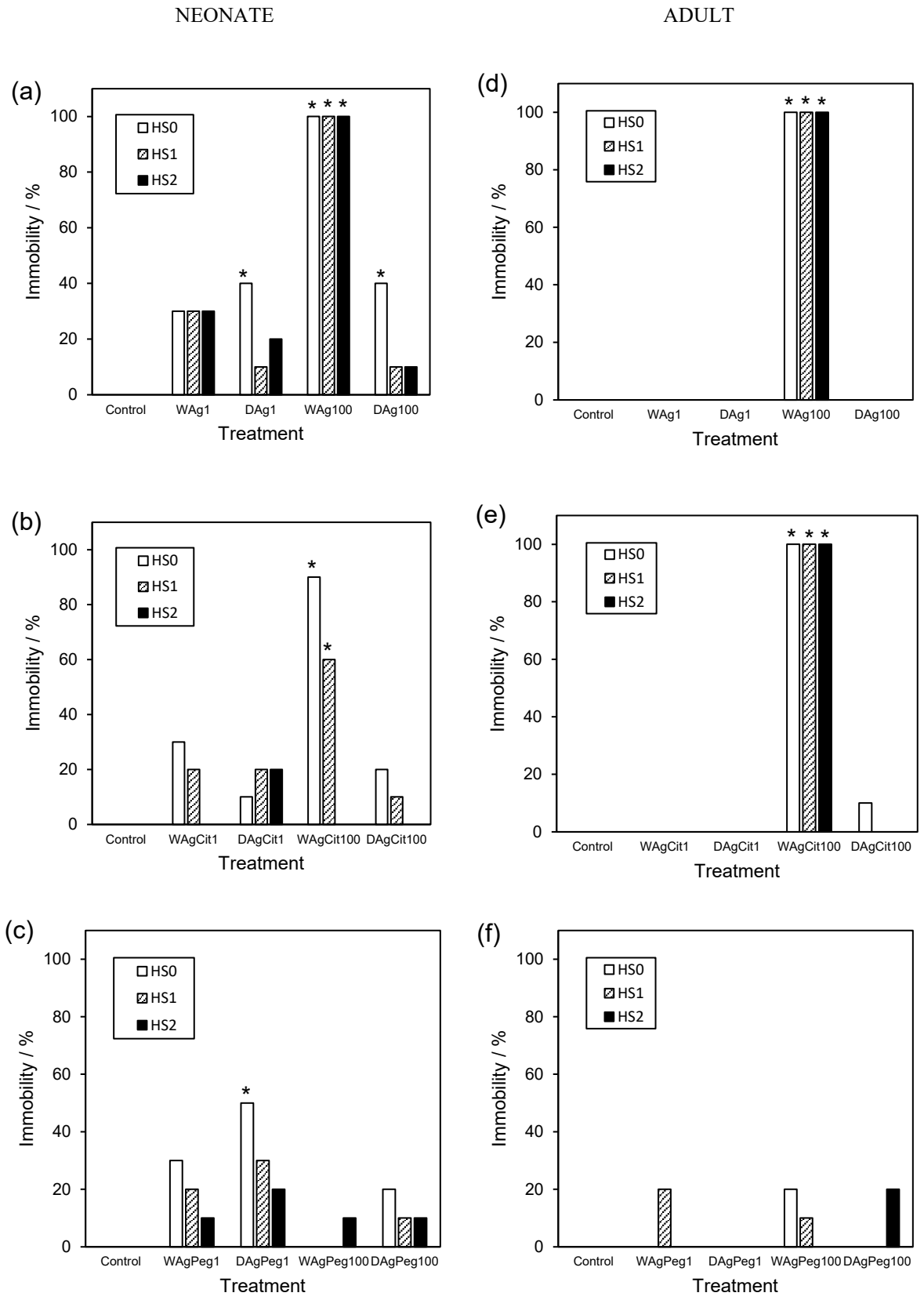

Figure 6. Waterborne (W) and dietborne (D) of *D. similis* exposition onto Ag^{+}, AgCit and AgPeg (100 $\mu g \cdot L^{-1}$) in absence (HS0) and presence of 1 and 10 $mg \cdot L^{-1}$ (HS1 and HS2, respectively) of humic substances. Experiments using Neonates (**a**–**c**), and Adults (**d**–**f**). The significant differences are indicated by ***** for $p \leq 0.05$.

3.4.3. Waterborne and Diet Exposition Comparison Test

The immobilization of *Daphnia similis* was recorded after 48 h of exposure to $AgNO_3$, AgCit and AgPeg exposition. According to the immobilization results organized in Figure 6, neonates (Figure 6a–c) are more sensitive compared to adults (Figure 6d–f), except when citrate nanosilver is directly dispersed in water (Figure 6b,e). At the highest exposure concentration, a contrasting effect was observed: no toxicity for neonates in the presence of HS2, but a clear toxic effect for adults across all humic substance treatments. This aligns with the understanding that early developmental stages of organisms are often more vulnerable to pollutant-induced damage than adult phases [54,55]. Further supporting this, toxicity bioassays across different life stages have revealed a pattern of increasing LC50 values with organism age, confirming neonates' heightened sensitivity compared to mature individuals (e.g., 7–8 days old), as indicated by significant statistical differences among the tested groups [56].

4. Conclusions

Our study lays the groundwork for elucidating the processes that determine the real-world exposure of two different thropic levels of aquatic organisms (*Daphnia similis* and *Raphidocelis subcapitata*) to various exposure scenarios (different concentrations of organic matter, Ag^+/AgNP, life-stage,). The toxicity of silver ions generally remained unchanged in the presence of natural organic matter. However, AgNP with different coatings (PEG and citrate) exhibited varying toxic effects. While AgPeg toxicity was observed, AgCit did not show toxicity at the same concentration. This suggests that AgPeg toxicity may be linked to the release of silver ions. The presence of natural organic matter increased silver bioaccumulation in algae, likely due to the formation of HS-Ag complexes. However, for citrate-coated nanoparticles, increased humic substance concentrations reduced silver bioaccumulation. In D. similis, the presence of humic substances exacerbated the toxicity of silver ions. For both types of AgNP, dissolution may contribute to their toxicity. Chronic exposure studies revealed toxic effects on Daphnia reproduction, even at permitted concentrations. Interestingly, humic substances may mitigate acute toxicity but can enhance chronic toxicity, as observed with low concentrations of AgPeg in the presence of humic substances. The replication of natural aquatic system characteristics through experimental assays aids in elucidating whether the stabilization of nanomaterials with HS or any other type of NOM will influence the toxicity of nanomaterials.

Supplementary Materials

The additional data and information can be downloaded at https://media.sciltp.com/articles/others/2507231530543913/ECCS-1151-Supplementary-Materials.pdf. Reference [57] is cited in the Supplementary Materials.

Author Contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: C.H.W., R.F.D., M.B. and A.H.R. Performed data acquisition: C.H.W. Provided administrative, technical and material support: R.F.D., M.B. and A.H.R. All authors have read and agreed to the published version of the manuscript.

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Data are available on request.

Conflicts of Interest

All authors declared that there is no conflict of interest.

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