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Antibacterial and Antibiofilm Potential of Microbial Polysaccharide Overlaid Zinc Oxide Nanoparticles and Selenium Nanowire

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Abstract: Here, we report on the synthesis of zinc oxide nanoparticles (ZnO NPs) and selenium nanowires (Se NWs) using microbial exopolysaccharides (EPS) as a mediator and then examine their antibacterial and ecotoxicity effects in vitro and in vivo, respectively. At 100 µg/mL, EPS, EPS-ZnO NPs, and EPS-Se NWs all exhibited potent in vitro antibacterial properties, drastically inhibiting the development of aquatic Gram⁽⁻⁾ pathogens. In addition, antibiofilm studies using a microscope revealed that EPS, EPS-ZnO NPs, and EPS-Se NWs at 75 µg/mL prevented biofilm development. Furthermore, the in vivo toxicity was carried out via *Danio rerio* embryos and *Ceriodaphnia cornuta*. *Danio rerio* embryos were determined at different time intervals (6 hpf, 12 hpf, 24 hpf and 48 hpf). The maximum survival rate (100%) was obtained in a control group. Correspondingly, EPS, EPS-ZnO NPs and EPS-Se NWs treated embryos showed a considerable survival rate with 93.3%, 86.7% and 77.2%, respectively, at 100 µg/mL for 48 hpf. The total mortality of *C. cornuta* was seen at 100 µg/mL, with 56.7% in EPS, 60.0% in EPS-ZnO NPs, and 70.0% in EPS-Se NWs. For *C. cornuta*, the LC₅₀ values for EPS, EPS-ZnO NPs, and EPS-Se NWs were 90.32, 81.99, and 62.99 µg/mL, respectively. Under a microscope, morphological alterations in *C. cornuta* were analyzed. After 24 h, an amount of dark substance was seen in the guts of *C. cornuta* exposed to 100 µg/mL, but in the control group, all of the living *C. cornuta* were swimming as usual. Our results show that EPS and EPS-ZnO NPs were less harmful than EPS-Se NWs, and that they were successfully employed to shield freshwater crustaceans from the toxins in aquatic environments.

Keywords: exopolysaccharides; nanotechnology; selenium nanowires; antibiofilm activity; aquatic pathogen; ecotoxicity assessment

1. Introduction

Researchers and industrialists have found several uses for nanoparticles (NPs) because of their important physiochemical properties [1–4]. Researchers are looking at the many qualities, origins, behavior, and negative repercussions of NPs because of their entry into aquatic systems as a result of excessive usage. There is no way to prevent nanomaterials from being released into water systems and affecting aquatic life. Around 7% of the

total volume of NPs synthesized [5–7] are released into aquatic ecosystems. Hence, it is crucial to assess the toxicological impacts of released NPs on aquatic organisms. In light of this, evaluating the toxicological effects of emitted NPs on aquatic species is essential. Despite this, there have been no clear recommendations or evidence for the long-term use of nanoparticles to protect aquatic life from the presumed hazards upon exposure to NPs [8]. The majority of toxicity investigations on NPs have been undertaken on titanium dioxide (31%), zinc oxide (17%), and silver NPs (13%), and environmental risk evaluations of these compounds are also considered as necessary research [9,10]. *Ceriodaphnia cornuta* and Zebrafish *Danio rerio* are two widely used and well-acknowledged model species for detecting the toxicity of chemicals and NPs [11] among aquatic creatures.

Bacterial exopolysaccharides (EPS) comprise natural bioactive compounds employed for food and biochemical and medical purposes, owing to their exclusive biological features [12–14]. Although they have multipurpose properties such as antitumor, antiviral, anti-inflammatory agents, antioxidant activity and immune-stimulating effects, they might also be utilized to treat infections caused by intracellular microorganisms because of their modest size. Exopolysaccharides and their encapsulated zinc oxide NPs and selenium nanowires have been reported previously [15–17]. Furthermore, there is less information on the antibiofilm activity of these Gram⁽⁻⁾ aquatic pathogens. Here, for the first time, we tested the effects of exopolysaccharides (EPS) from the probiotic *Bacillus licheniformis* on aquatic species, both alone and in combination with zinc oxide nanoparticles (EPS-ZnO NPs) and selenium nanowires (EPS-Se NWs). Additionally, the potential of EPS, EPS-ZnO NPs, and EPS-Se NWs as antibacterial and antibiofilm agents against Gram⁽⁻⁾ aquatic pathogens was investigated.

2. Materials and Methods

2.1. Sample Preparation

EPS from *B. licheniformis* Dab11 was extracted using the previously reported technique [15]. Briefly, *B. licheniformis* was grown in 500 mL of nutritional medium at 37 °C for 72 h. In addition, the cells were separated by centrifugation (8000 × g for 10 min) and heated (100 °C for 30 min to inactivate the enzymes). The EPS-containing supernatant fluid was precipitated by adding three liters of ice-cold, 95% ethanol at 20 °C. After centrifugation (8000 × g for 10 min), it was stored at 4 °C for 36 h. Finally, the pellet was desiccated and kept at 30 °C.

Additionally, zinc oxide nanoparticles (ZnO NPs) and selenium nanowires (Se NWs) were bio-synthesized using EPS, as detailed in recent research [16,17].

The EPS-ZnO NPs were synthesized by adding 0.5 g of EPS crude powder to an aqueous solution (0.02 M) of zinc acetate and NaOH (2 M) while stirring vigorously at 37 °C. The mixture was centrifuged at 8000 × g for 10 min and washed thrice. The precipitate was dried in an oven set to 70 °C.

The EPS-Se NWs were synthesized by combining crude EPS powder (0.5 g) with sodium selenite (0.1 M) in an aqueous solution, which was then maintained at 37 °C under vigorous magnetic stirring for 10 min before being centrifuged at 8000 × g and rinsed three times with d.H₂O, constraining the pellet and drying it out at 75 °C. The EPS, EPS-ZnO NPs, and EPS-Se NWs that were successfully preserved were then used in the experiments.

2.2. Microbial Activity

2.2.1. Minimum Inhibitory Concentration (MIC) of EPS, EPS-Zn O NPs and EPS-Se NWs

The MIC of EPS, EPS-ZnO NPs and EPS-Se NWs were evaluated against Gram⁽⁻⁾ aquatic pathogens of *V. harveyi* (HQ693276), *V. alginolyticus* (ATCC 17749), *V. parahaemolyticus* DahV2 (HQ693275.1) and *A. hydrophila* (ATCC 7966) by resazurin microtiter-plate assay [18,19]. Samples between 10–100 µg/mL were added to 100 µL of nutrient broth (HiMedia, India). Then, 10 µL of resazurin solvent (0.25 g dissolved in 30 mL of 1X PBS) was added. After that, a microbial suspension (10 µL; 5 × 10⁶ CFU per mL) was added and cultured at 37 °C for 24 h. As a reference point, we used a PBS solution with a pH of 7.4.

Once incubation was complete, the result was assessed visibly from blue to pink, indicating the growth of bacteria (control) and retaining blue color, which signals the suppression of culture growth. Then, MIC was estimated

2.2.2. Live/Dead Assay in the Presence of EPS, EPS-ZnO NPs and EPS-Se NWs

Live/Dead assay was determined using the Bac/Light kit (Invitrogen, USA) through a previously available technique [20,21]. Briefly, using a 24-well plate, inoculate the nutrient medium with a 12-h-old Gram⁽⁻⁾ aquatic bacterial culture (50 μ L; 1×10^6 CFU/mL), then 75 μ g/mL of EPS, EPS-ZnO NPs, and EPS-Se NWs were added and incubated at 37 °C for 60 min. After obtaining a bacterial cell pellet by centrifugation (7000 $\times$ g for 10 min at 4 °C), the pellet was washed with PBS, stained with STYO[®]9 (10 μ L) and propidium iodide (20 μ L), and finally visualized using a fluorescent microscope. Confocal Laser Scanning Microscopy (CLSM) examination was done after a 15-min incubation at 37 °C in the dark.

2.2.3. Antibacterial Activity of EPS, EPS-ZnO NPs and EPS-Se NWs

The Agar well diffusion method of EPS, EPS-ZnO NPs and EPS-Se NWs was assessed for Gram⁽⁻⁾ aquatic pathogens [22]. A Gram⁽⁻⁾ aquatic culture of each bacterium was swabbed onto an LB agar plate after 12 h (1×10^6 CFU per mL; 100 μ L), and wells of 6 mm in diameter were drilled for them using a cork borer. The concentrations of EPS, EPS-ZnO NPs, and EPS-Se NWs in the test samples were 25, 50, 75, and 100 μ g/mL; D.H₂O (100 μ L) was used as the negative control. After incubation at 37 °C for 24 h, the size of the zone of inhibition was measured in millimeters, and the data were reported as the mean $\pm$ standard deviation.

2.2.4. Evaluation of EPS, EPS-ZnO NPs, and EPS-Se NWs for Antibiofilm Activity

To evaluate the influence of EPS, EPS-ZnO NPs and EPS-Se NWs to arrest the biofilm formation on Gram⁽⁻⁾ we used aquatic pathogens through 24-well microtiter plate techniques [23,24]. To sum up, Gram⁽⁻⁾ aquatic pathogens were given 1.5 mL of nutrient broth containing 75 μ g/mL of EPS, EPS-ZnO NPs, and EPS-Se NWs, and allowed to develop a biofilm on 1 $\times$ 1 cm glass pieces. At 40 $\times$ magnification using a light microscope (U-RFLT50), we saw samples stained with 0.4% acridine orange that had been incubated for 48 h at 37 °C. The supplementary sets were also made the same way and stained with 0.1% crystal violet before being examined under CLSM at 20 $\times$ magnification.

2.3. Toxicity Effect on Aquatic Organisms

2.3.1. Zebrafish (*Danio rerio*) Embryo Assay

Danio rerio, a kind of aquatic vertebrate (length: 3.5 cm; weight: 0.8 g), was acquired from a regional hatchery; the tank held 25 L of water, and the fish were fed artemia culture three times daily. The pH of the water was measured to be 7.5 ± 0.5 , the dissolved oxygen to be 6.5 ± 0.5 mg/L, the temperature to be 26.5 ± 0.5 °C, and the duration of the light to be 10.50 h. Afterward, embryo production was attained after the breeding of fish. For breeding, 2 males to 1 female were placed in a breeding setup box with a net partition and kept at 12-h light/12-h dark; the partition was removed after 24 hrs and the fish were allowed to spawn the eggs for 2 h. Thenceforth, the viable eggs were collected with a fine fry net, transferred to the tank water, and their embryos examined using a dissecting microscope at low magnification. Then, the healthy embryos were transferred to HF medium and rinsed three times; any cloudy or ruptured embryo were discarded.

The toxicity of embryos was studied by following standard protocol [25] with some modifications. In brief, the embryos were exposed to EPS, EPS-ZnO NPs and EPS-Se NWs at 20, 40, 60, 80, and 100 μ g/mL doses in a 24-well microtitre plate. Twelve embryos were utilized between the sample and the control, according to OECD [26] guidelines. Every sample was tested three times and kept in an incubator at 27 ± 1 °C. Six, twelve, twenty-four, and forty-eight hours after treatment, morphological and toxic effects were examined under

a light microscope. The deformity was also evaluated under a light microscope. Death rates were calculated using the live births percentage among treated embryos.

2.3.2. *Ceriodaphnia cornuta* Assay

An aquatic invertebrate, *Ceriodaphnia cornuta*, was raised from stock culture retained in our research laboratory. According to criteria [27], *C. cornuta* was cultured in freshwater, which was sustained in 50 animal/L and fed daily with 6×10^7 cells/mL of green algae *Chlorella vulgaris*/L (20 mL). For this, total hardness: 80–100 mg L⁻¹ as CaCO₃; Alkalinity: 57–64 as CaCO₃; pH: 7.9 ± 0.2 ; photoperiod: 16 h light/8 h dark; and water renewed twice a week [28,29].

The toxicity of EPS, EPS-ZnO NPs and EPS-Se NWs was determined on *C. cornuta* at a dose of 20–100 µg/mL through the following protocol [30] with minor changes. Twenty neonates (age < 24 h) were taken into a 24-well plate with 10 animals in each well and without feeding during this experimental setup. After 24 h, the mortality (%) and LC₅₀ of each treatment were determined. Furthermore, the physisorption and internalization of EPS, EPS-ZnO NPs and EPS-Se NWs on *C. cornuta* were noticed under a light microscope at 40× magnification.

2.4. Statistical Analysis

Results are presented as means standard deviations using a one-way analysis of variance (ANOVA) and Tukey's HSD test in SPSS version 21. Chi-square testing and probit analysis were used to determine the LC₅₀ and LC₉₀. To determine whether or not there were significant changes between the control and treatment groups, we used a limit of $p < 0.05$ in all analyses

3. Results

3.1. Microbial Activity

3.1.1. MIC of EPS, EPS-ZnO NPs and EPS-Se NWs

The MIC for EPS, EPS- ZnO NPs and EPS-Se NWs against aquatic pathogens *V. harveyi*, *V. alginolyticus*, *A. hydrophila* and *V. parahaemolyticus* was evaluated by resazurin microtiter-plate assay (Figure 1). EPS revealed MIC at *V. harveyi*, (50 µg/mL), *V. alginolyticus* (40 µg/mL), *A. hydrophila* and *V. parahaemolyticus* (30 µg/mL). In EPS- ZnO NPs at *V. harveyi*, (40 µg/mL), *V. alginolyticus*, *A. hydrophila* and *V. parahaemolyticus* (30 µg/mL). EPS-Se NWs revealed MIC at 30 µg/mL for *V. harveyi*, *V. alginolyticus*, *A. hydrophila* and *V. parahaemolyticus*. After 24 h, the color changed from blue to pink in all control wells, but there seemed to be no change in the EPS, EPS-ZnO NPs, and EPS-Se NWs wells. Therefore, it failed to proliferate, demonstrating the pathogen's insensitivity.

3.1.2. Live/Dead Assay in the Presence of EPS, EPS-ZnO NPs and EPS-Se NWs

Figure 2 shows the results of CLSM analysis of live/dead bacterial cells stained with BacLight fluorescent stains; the green fluorescence represents viable cells, while the red fluorescence represents cells that have already died. It was shown that EPS, EPS-ZnO NPs, and EPS-Se NWs seemed to have effective bactericidal effects at 75 µg/mL after being used to treat aquatic infections, with a significantly lower number of live cells being seen in the treated samples compared to the control samples.

3.1.3. Antibacterial Activity of EPS, EPS-ZnO NPs and EPS-Se NWs

The EPS, EPS-ZnO NPs, and EPS-Se NWs were tested for their ability to inhibit the growth of *V. harveyi*, *V. alginolyticus*, *A. hydrophila*, and *V. parahaemolyticus* using the agar well diffusion technique. Results are shown in Table 1, and there was no inhibition in the control well. With 100µg/mL, a zone of inhibition of EPS was revealed for *V. harveyi*, (8.23 ± 0.87), *V. alginolyticus* (9.3 ± 0.6), *A. hydrophila* (9.8 ± 0.3) and *V. parahaemolyticus* (9.6 ± 0.3) while EPS- ZnO NPs was exhibited for *V. harveyi*, (9.3 ± 0.82), *V. alginolyticus* (9.5 ± 0.3), *A. hydrophila* (9.9 ± 0.1) and *V. parahaemolyticus* (9.7 ± 0.3). Moreover, EPS-Se

NWs were exhibited for *V. harveyi*, (8.23 ± 08.7), *V. alginolyticus* (8.93 ± 0.64), *A. hydrophila* (9.4 ± 0.60) and *V. parahaemolyticus* (9.2 ± 0.71). Overall, EPS, EPS-ZnO NPs and EPS-Se NWs showed greater inhibition on *A. hydrophila* and *V. parahaemolyticus* at $100 \mu\text{g/mL}$ when compared to *V. harveyi*, *V. alginolyticus*.

3.1.4. Antibiofilm Activity of EPS, EPS-ZnO NPs and EPS-Se NWs

Figures 3 and 4 displayed the CLSM and light microscopy images of antibiofilm action with EPS, EPS-ZnO NPs and EPS-Se NWs against aquatic pathogens (*V. harveyi*, *V. alginolyticus*, *A. hydrophila* and *V. parahaemolyticus*). The control biofilm (without EPS, EPS-ZnO NPs, and EPS-Se NWs) had a greater surface colonization for all bacterial strains than the treated biofilms (EPS, EPS-ZnO NPs, and EPS-Se NWs), which displayed poor adherence and biofilm erosion at $75 \mu\text{g/mL}$.

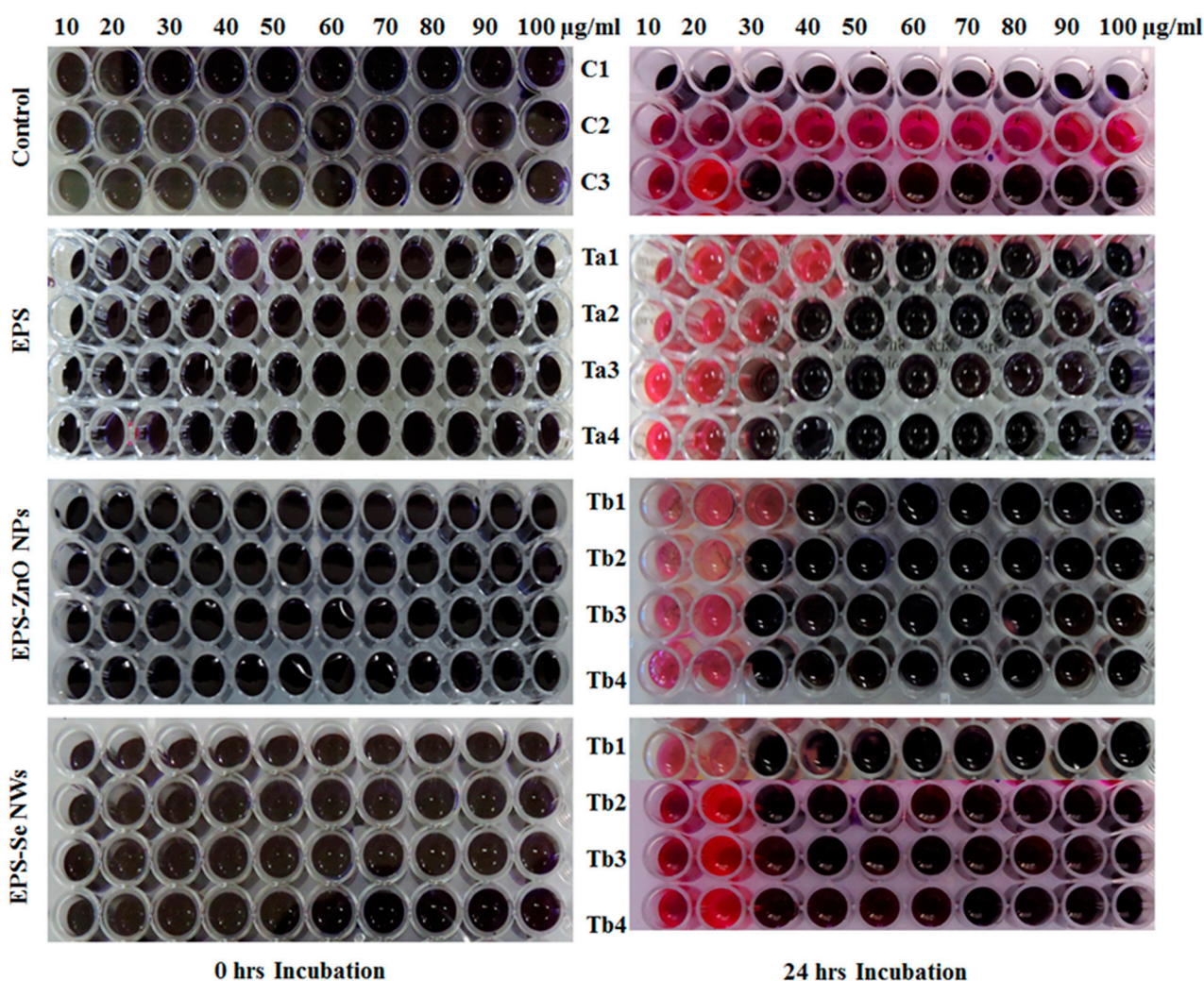


Figure 1. The minimum inhibitory concentration of EPS and EPS-ZnO NPs against aquatic pathogens by resazurin microtitre plate method. After 24 h, the pink color designates the growth and blue indicates inhibition of growth; the test organisms were *V. harveyi* (HQ693276), *V. alginolyticus* (ATCC 17749), *V. parahaemolyticus* DahV2 (HQ693275.1) and *A. hydrophila* (ATCC 7966). In control, C1—sterility control without bacteria (test compound in broth + saline + indicator); C2—negative control without drug (test organisms +broth + indicator); C3—positive control (tetracycline + broth + indicator + test organisms); Ta1–Ta4 and Tb1–Tb4 (test compound EPS and EPS-ZnO NPs + broth + indicator + test organisms).

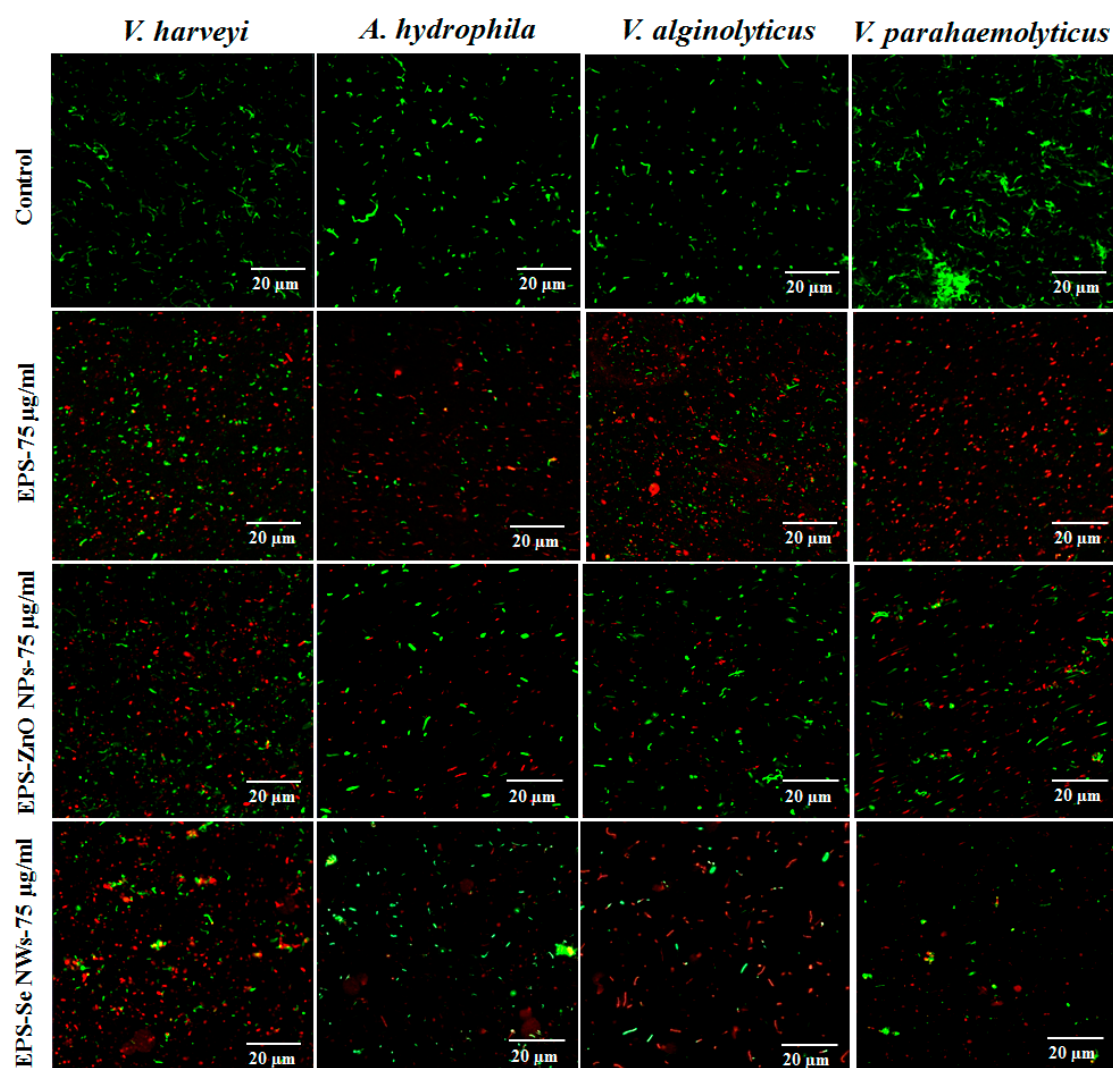


Figure 2. BacLight live and dead assay of EPS, EPS-ZnO NPs and EPS-Se NWs tested against aquatic pathogens.

Table 1. Toxicity effect of EPS, EPS-ZnO NPs and EPS-Se NWs against *Ceriodaphnia cornuta*. No mortality was observed in control.

Materials	Concentration (µg/mL)	Mortality (%) ± SD	LC ₅₀ (95% LCL-UCL) (µg/mL)	χ^2 (d.f. = 4)
Exopolysaccharide (EPS) from probiotic <i>B. licheniformis</i> Dahb1	20 40 60 80 100	13.3 ± 0.58 23.3 ± 0.58 30.0 ± 1.00 43.3 ± 1.53 56.7 ± 1.15	90.32 (81.38–103.58)	0.350 n.s
Exopolysaccharides coated zinc oxide nanoparticles (EPS-ZnO NPs)	20 40 60 80 100	20.0 ± 1.00 26.7 ± 0.58 36.7 ± 0.58 50.0 ± 1.00 60.0 ± 1.00	81.99 (73.26–94.43)	0.205 n.s
Exopolysaccharides capped selenium nanowires (EPS-Se NWs)	20 40 60 80 100	33.3 ± 2.52 36.7 ± 0.58 50.0 ± 1.00 53.3 ± 0.58 70.0 ± 1.00	62.99 (53.05–73.70)	2.025 n.s

n.s. = not significant ($p > 0.05$).

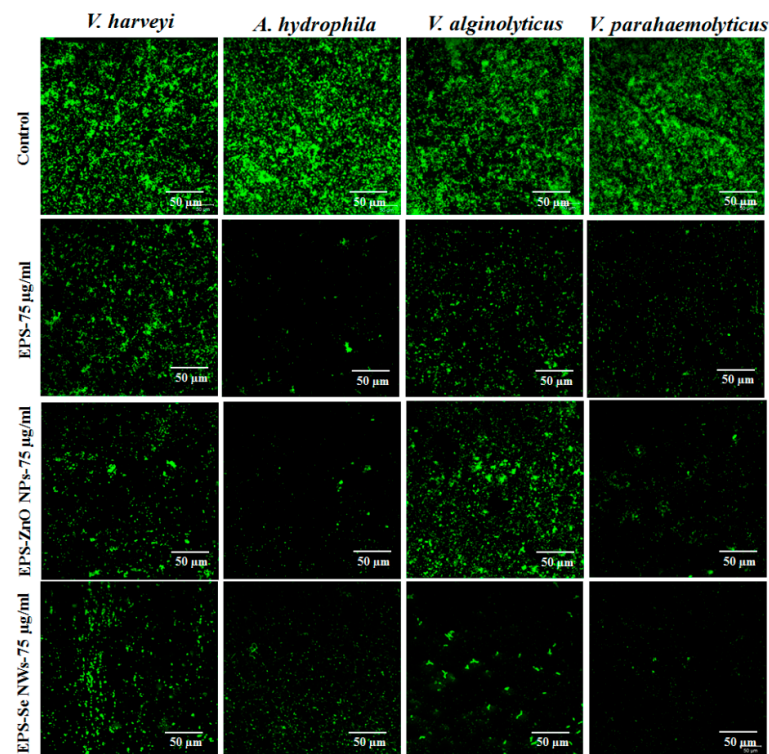


Figure 3. Confocal laser scanning microscopy images of antibiofilm activity of EPS, EPS-ZnO NPs and EPS-Se NWs tested against aquatic pathogen.

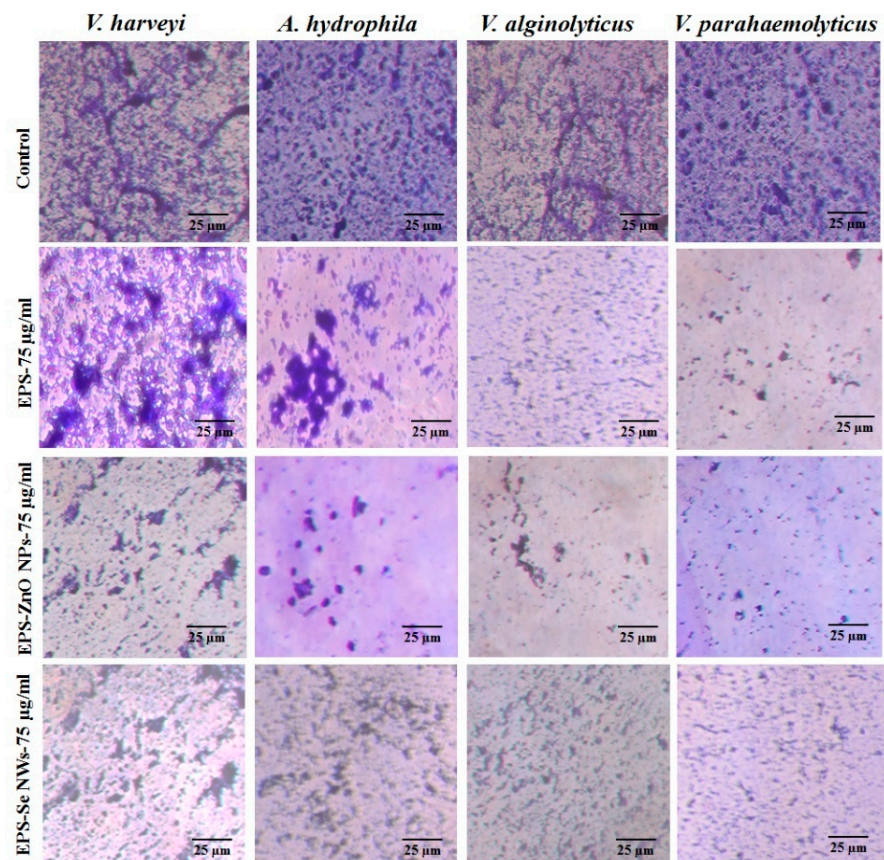


Figure 4. Light microscopy images of antibiofilm activity of EPS, EPS-ZnO NPs and EPS-Se NWs tested against aquatic pathogens.

3.2. Toxicity Effect on Aquatic Organisms

3.2.1. Zebrafish (*Danio rerio*) Embryo Assay

The toxicity effect was determined using EPS, EPS-ZnO NPs and EPS-Se NWs on *Danio rerio* at various concentrations (Figure 5). The survival rates of embryos at 6, 12, 24, and 48 h were determined. The maximum survival rate (100%) was obtained in a control group. Correspondingly, EPS, EPS-ZnO NPs and EPS-Se NWs treated embryos showed a considerable survival rate with 93.3%, 86.7% and 77.2%, respectively, at 100 $\mu\text{g/mL}$ for 48 hpf. The toxic effect of EPS, EPS-ZnO NPs and EPS-Se NWs treated embryos was found to depend on dose and time of exposure. Compared to EPS-Se NWs-treated embryos at 48 hpf, those exposed to EPS and EPS-ZnO NPs at 100 $\mu\text{g/mL}$ showed no signs of death, delay in hatching, or morphological deformations.

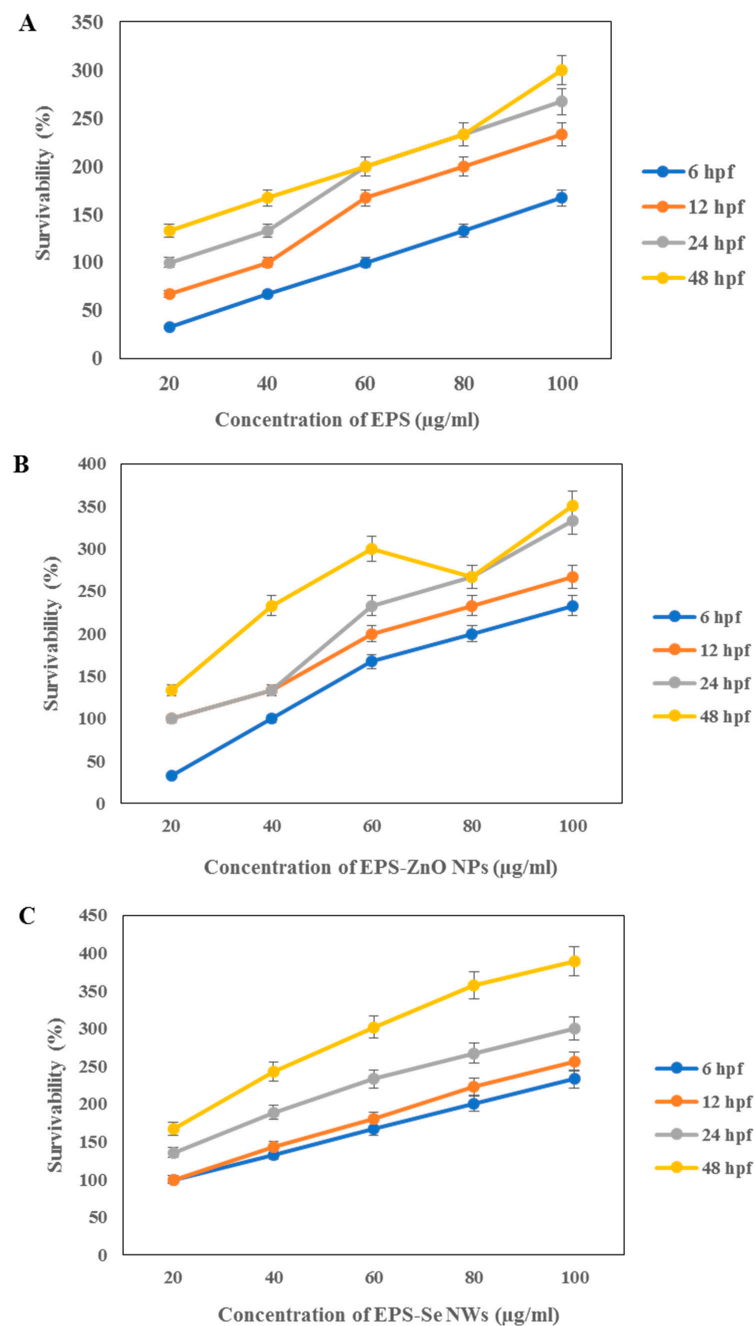


Figure 5. Survivability of Zebrafish (*Danio rerio*) embryo treated against EPS (A), EPS-ZnO NPs (B) and EPS-Se NWs (C) up to 48 hpf.

3.2.2. *Ceriodaphnia cornuta* Assay

The toxicity of EPS, EPS-ZnO NPs, and EPS-Se NWs was tested in *Ceriodaphnia cornuta* at different doses. Maximum *C. cornuta* mortality was seen across all tested materials, with results showing 56.7% mortality at 100 µg/mL in EPS, 60.0 in EPS-ZnO NPs, and 70.0 in EPS-Se NWs. The LC₅₀ values of EPS EPS-ZnO NPs and EPS-Se NWs against *C. cornuta* were 90.32, 81.99 and 62.99 µg/mL, respectively (Table 2).

Table 2. In vitro antibacterial activity of EPS, EPS-ZnO NPs and EPS-Se NWs tested against aquatic pathogens.

Materials	Pathogens	25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
EPS	<i>V. alginolyticus</i>	2.9 ± 0.4	5.9 ± 1.7	7.2 ± 1.5	9.3 ± 0.6
	<i>A. hydrophila</i>	3.7 ± 0.2	5.6 ± 1.3	8.2 ± 1.3	9.8 ± 0.3
	<i>V. parahaemolyticus</i>	3.0 ± 0.2	5.3 ± 0.8	7.7 ± 0.7	9.6 ± 0.3
	<i>V. harveyi</i> (HQ693276)	3.9 ± 0.2	5.2 ± 1.1	8.2 ± 0.4	8.2 ± 0.8
	<i>V. alginolyticus</i>	3.2 ± 0.3	6.6 ± 0.5	8.3 ± 0.6	9.5 ± 0.3
EPS-ZnO NPs	<i>A. hydrophila</i>	4.7 ± 0.2	7.6 ± 1.2	9.2 ± 0.8	9.9 ± 0.1
	<i>V. parahaemolyticus</i>	3.9 ± 0.3	7.0 ± 0.3	8.7 ± 0.2	9.7 ± 0.3
	<i>V. harveyi</i> (HQ693276)	3.4 ± 1.0	5.5 ± 1.0	5.7 ± 1.0	9.3 ± 0.8
	<i>V. alginolyticus</i>	2.6 ± 0.7	3.3 ± 0.3	6.3 ± 2.3	8.9 ± 0.6
	<i>A. hydrophila</i>	3.0 ± 0.3	5.9 ± 1.6	7.2 ± 1.5	9.4 ± 0.6
EPS-Se NWs	<i>V. parahaemolyticus</i>	3.07 ± 0.1	4.8 ± 0.3	6.5 ± 1.1	9.2 ± 0.7
	<i>V. harveyi</i> (HQ693276)	1.2 ± 0.2	2.5 ± 0.6	5.1 ± 0.9	8.2 ± 0.8

Under a microscope, morphological changes in *C. cornuta* were studied (Figure 6). When exposed to EPS, EPS-ZnO NPs, and EPS-Se NWs for 24 h, the anomalies were found at 100 µg/mL concentrations. *C. cornuta*-treated samples showed a significant increase in the quantity of black material in the digestive system. In the control group, the live *C. cornuta* swam properly. Additionally, differences were seen after administering EPS, EPS-ZnO NPs, and EPS-Se NWs, including bubble formation surrounding the body, intestinal thickness and blackening, and carapace rupture in *C. cornuta*. Our findings suggest that EPS and EPS-ZnO nanoparticles were less hazardous than EPS-Se NWs when tested on *C. cornuta*.

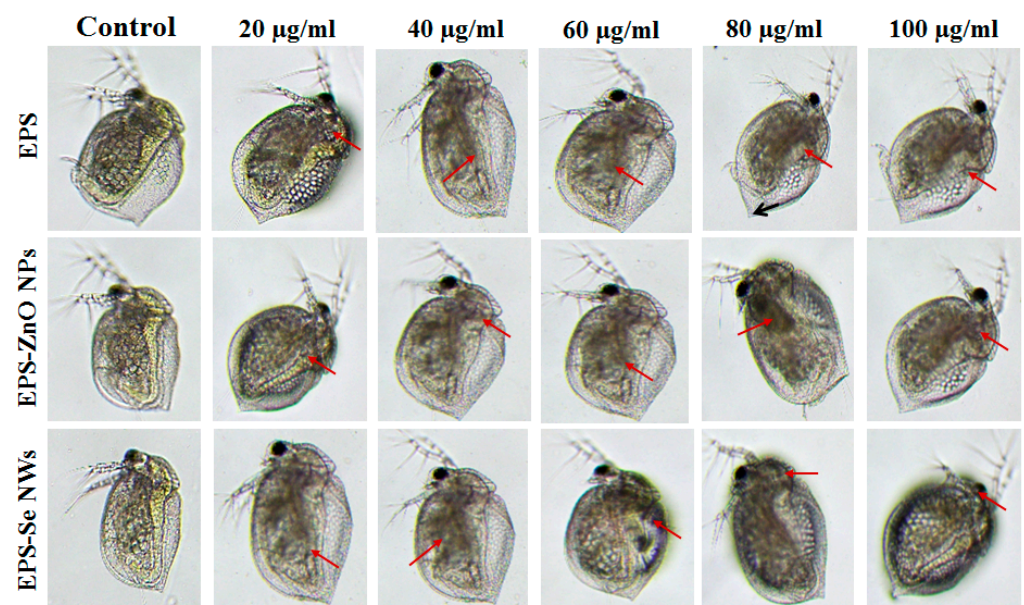


Figure 6. Light microscopy showing toxicity effect on *Ceriodaphnia cornuta* exposed to EPS, EPS-ZnO NPs and EPS-Se NWs. Arrows indicate morphological changes.

4. Discussion

Exopolysaccharides from probiotic *B. licheniformis* (EPS) and its mediated zinc oxide nanoparticle (EPS-ZnO NPs) and selenium nanowires (EPS-Se NWs) were evaluated for the first time for their toxicity to aquatic organisms. In addition, the antibiofilm ability of EPS, EPS-ZnO NPs, and EPS-Se NWs against Gram⁽⁻⁾ aquatic pathogens that cause illness was investigated. Outcomes reveal maximum growth inhibition in *V. parahaemolyticus* DahV2 and *A. hydrophila* over *V. harveyi*, *V. alginolyticus* bacteria at 100 µg/mL of EPS, EPS-ZnO NPs and EPS-Se NWs. Our results are consistent with recent research on *E. faecalis*-produced Se NPs, which has shown that these particles are efficient against *E. coli*, *B. subtilis*, *P. aeruginosa*, and *S. aureus* [31,32]. The live/dead cells experiment shows that the Gram⁽⁻⁾ aquatic pathogens are arrested by EPS, EPS-ZnO NPs, and EPS-Se NWs at a 75 µg/mL concentration. Previous studies have suggested that propidium iodide (PI) might bond with DNA molecules to boost fluorescence. Since it is unable to traverse the membrane, it is effectively blocked from entering living cells [20,21]. Mahendran et al. [33] have stated that exopolysaccharides could effectually interact with pathogens relying on their cell-membrane penetrability, inducing cell death to trigger ROS production.

Similarly, Gram⁽⁻⁾ biofilm development was inhibited and poor adhesion was seen when the microorganisms were exposed to EPS, EPS-ZnO NPs, and EPS-Se NWs at 75 µg/mL, demonstrating antibiofilm activity. These findings are consistent with those of other studies on antibiofilm activity [34–36]. Zhang et al. [37] hypothesized that these results are due to ZnO NPs greater capacity to penetrate the membrane of bacteria than EPS-ZnO NPs. The electrostatic repulsion between the bacterial surface and the NPs was shown to trigger the antibacterial effect by Stoimenov et al. [38]. Our results corroborate those of Tran and Webster [39], who showed that Se NPs suppressed *S. aureus* biofilm development after 5 h of exposure in EPS-Se NWs. In a similar vein, both Se NWs and NPs were able to slow the development of biofilms [40–43].

An animal model, *Danio rerio* (embryo), may be used to investigate the toxicity of bacterial derivatives [44]. The current study found that at 48 h after fertilization, embryos treated with either synthetic EPS or EPS-ZnO NPs (at a concentration of 100 µg/mL) exhibited no significant increases in mortality, delayed hatching or morphological defects. High concentrations (100 ppm) are responsible for the vilified effect, forming a sticky coating surrounding embryos, ultimately leading to death [45]. Additionally, using such a concentration from the previously described *A. squamosa* increased mortality and reduced survival [46].

Maximum *C. cornuta* mortality was calculated to be 56.7% in EPS, 60.0% in EPS-ZnO NPs, and 70.0% in EPS-Se NWs at 100 µg/mL. Against *C. cornuta*, the LC₅₀ values for EPS, EPS-ZnO NPs, and EPS-Se NWs were 90.32, 81.99, and 62.99 µg/mL, respectively. Previous research has shown that when *Daphnia* are exposed to high concentrations of colloidal solutions, their behavior changes, including higher swimming speeds and a tendency to migrate to the top of the solution [47]. The current investigation corroborated the results of Shanthi et al. [48], who discovered that AgNO₃ resulted in a greater death rate for *C. cornuta* than BLCFE-AgNPs. At doses of up to 6400 mg Zn/kg d.w., neither ZnO-NPs nor non-nano ZnO affected *Folsomia candida*, as reported by Kool et al. [49]. Manzo et al. [50] found that nano ZnO was more dangerous than ionic zinc. Some have speculated that the more significant toxicity of nano ZnO was attributable to the nano state's peculiar physicochemical features compared to the bulk material. Plb-ZnO NPs caused a 100% mortality rate in *C. cornuta* neonates at 160 µg/L (LC₅₀: 28 µg/L), as reported by Vijayakumar et al. [51]. Heinlaan et al. [52] determined an LC₅₀ for micro ZnO of 1.9 mg/L, which is consistent with their results. TiO₂ (100–140 nm) showed an EC₅₀ larger than 100 mg L⁻¹ on *D. magna* over 48 h, as determined by Warheit et al. [53]. Then, Lovern and Klapper [54] demonstrated that TiO₂ NPs caused the death of *D. magna* at varying concentrations (LC₅₀ = 5.5 mg L⁻¹). Adam et al. [55] found a significant decrease in neonates and an increase in zinc accumulation with increasing dosages of ZnO and CuO NPs.

On the other hand, chemical toxicants are often found in aquatic environments, which are very hazardous to water systems and disturb aquatic life. Plasticizer effluents, such as di(2-Ethylhexyl) phthalate (DEHP) and tris (2-butoxy ethyl) phosphate (TBOEP), have both acute and chronic effects. Thus, several pieces of literature, including, for instance, acute tests, show the 48 h-EC₅₀/48 h-LC₅₀ of DEHP to freshly hatch neonates of *D. magna* ranged between 160 and 3310 µg DEHP L⁻¹ [56–59]. Nonetheless, the 48-EC₅₀ and 48-h-LC₅₀ values for TBOEP against *D. magna* were very high, 38 mg L⁻¹ and 147 mg L⁻¹, respectively [60,61]. In *D. magna*, sublethal doses of DEHP and TBOEP may significantly affect genetic and cellular responses [62,63]. In 14-day chronic tests, DEHP and TBOEP significantly decreased the survival of *C. cornuta* at 100 µg L⁻¹ in the water of the Mekong River [64]. Yuan Liu et al. [65] observed that wastewater influent to *D. magna* and zebrafish embryos demonstrated severe toxicity (100% mortality) within 24 h, with characterization indicating that organics, metals, and volatiles all contributed to the toxicity. Ved Prakash et al. [66] reported that waterborne exposure induced developmental toxicity of 4-methyl benzylidene camphor (4-MBC) with a 96 h LC₅₀ of 2.71 mg/L. At the same time, embryos exposed to sub-lethal concentrations (50 and 500 µg/L) experienced a significant delay in hatching rate, heart rate, reduced larval length, and restricted hatchlings motility in addition to axial curvature. The results of scientific literature and studies indicate that nanoparticles are less dangerous than chemical substances. *C. cornuta* treated with EPS or EPS-ZnO NPs was much less hazardous than *C. cornuta* treated with EPS-Se NWs.

5. Conclusions

The results reveal that EPS, EPS-ZnO NPs and EPS-Se NWs proved to be less toxic for aquatic invertebrate and vertebrate organisms. Therefore, they displayed effective antibacterial action towards Gram⁽⁻⁾ aquatic pathogens. Overall, the study highlights that EPS, EPS-ZnO NPs, and EPS-Se NWs may be used to develop effective antimicrobial agents and protect freshwater crustaceans from harmful effects in aquatic environments.

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