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Influence of NOM on the Stability of Zinc Oxide Nanoparticles in Ecotoxicity Tests

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Abstract: Nanomaterials are known to aggregate in the presence of ions. Similarly, the aggregation of zinc oxide nanoparticles (ZnO NPs) exposed to various ions such as sodium chloride and calcium chloride in water systems increases with the ionic strength. Therefore, for accurate toxicity studies, it is necessary to conduct a test using natural organic matters (NOMs) as additional dispersants that strengthen stability with increased repulsive forces. The three types of ecotoxicity tests based on the dispersion stability test using NOM showed that the toxicities of the three test samples decreased in the presence of NOM. To determine how NOM improved dispersion and reduced toxicities, we analyzed the ionization degree of ZnO NPs with and without NOM and found that the solubility was below 2 mg/L with a negligible change over time, implying that the ionization effect was low. The absolute value of the surface charge of particles increased in the presence of NOM, resulting in increased repulsive electrostatic forces and steric hindrance, causing less aggregation and more dispersion. Additionally, although the NOM used in the test is considered an effective dispersant that does not have a toxicological effect on aquatic organisms, the presence of NOM resulted in reduced toxicities and should be further investigated to establish it as a standard test method.

Keywords: nanoparticles; zinc oxide; ecotoxicity; natural organic matters

1. Introduction

In recent years, international communities such as Organization for Economic Co-operation and Development (OECD) and the European Union (EU) have been working on test guidelines regarding the physico-chemical properties and hazards of manufactured nanomaterials (MNs), while internationally expanding the scope of MNs to advanced materials [1–3]. As nanomaterials are being increasingly used, there has been a growing concern regarding environmental safety [4].

Of all the MNs, metallic nanomaterials, in particular, show aggregation resulting from increased ionic strength of the test medium and owing to the presence of various ionic substances, consequently hindering accurate toxicity tests [5,6]. From an environmental perspective, the amount of nanomaterials entering water systems decreases with nanomaterial aggregation and precipitation, which consequently partially increases the exposure to the surrounding area where aggregation and precipitation occur. By contrast, improved particle mobility will facilitate dispersion in water systems over a long range, and therefore, the possibility of long-distance pollution should also be considered [7]. While inorganic nanomaterials are well-aggregated in the presence of ions, ZnO nanomaterials exposed to various ions such as sodium chloride and calcium chloride in water systems increase aggregation with the increase

in the strength of ions [8,9]. In addition, as the repulsive force varies depending on the type of ions, the different types of ions exposed will show different aggregation tendencies for the particles [10]. Therefore, for accurate toxicity studies, it is necessary to conduct a test using additional dispersants that strengthen stability with increased repulsive force.

OECD has suggested the development of acute toxicity guidelines to conduct toxicity evaluation of the soluble chemicals. However, for the insoluble nanomaterials with unique physicochemical and toxic properties, the applicability of the test guidelines (TGs) should be further considered. OECD TG 318 suggested a testing method for improving dispersion by adding natural organic matters (NOMs) as a dispersant, and recommended that the concentrations of the stock solution be determined and the concentrations of the test media be maintained through an hourly dispersion measurement of the nanomaterials [11]. In a previous study, the method of dispersing nanomaterials using ultrasonic waves and dispersants was used to address the problem of aggregation. However, excessive exposure to ultrasonic waves can lead to the oxidized nanomaterials generating increased amounts of ions, thereby increasing the oxidative stress of the exposed species, as well as the toxic effects of the dispersants that are used for surface modification of the nanomaterials [12].

To reduce these adverse effects, several studies have been undertaken using NOM, which are ubiquitous complex mixtures of organic matters that exist in nature and can improve the dispersion of nanoparticles (NPs). However, compared to Ag NPs, other metallic nanomaterials such as ZnO NPs have not been widely investigated to confirm their toxic effects in the presence or absence of NOM. According to existing research, when NOMs are used as dispersants, the toxicities of Ag NPs are likely to be reduced. Kennedy et al. [13] also showed that the presence of NOM resulted in a 2.5–6.7 fold decrease in toxicities for 20 nm Ag NPs and a 2.7–3.1 fold decrease in toxicities for 100 nm Ag NPs. Furthermore, Gao et al. [14] and Kim et al. [15] proved that NOM reduced the toxicities of Ag NPs, stating that the possible causes of the reduced toxicities were the different ionization mechanisms of Ag NPs with and without NOM.

As shown in Table 1, for ZnO NPs in the absence of NOM, half effective concentration (EC_{50}) (72 h) for algae ranged from 37 to 68 μg/L [16,17], whereas EC/LC_{50} (48 h) for water fleas ranged from 0.62 to 7.5 mg/L [18–26]. LC_{50} (96 h) for fish was in the range of 1.55 to 3.97 mg/L [27,28], indicating a significant difference of up to 12 times depending on the particle size and test medium. Cupi et al. [20] reported that the toxicity of ZnO to water fleas increased from 4.7 to 2.2 mg/L (EC_{50}). The ionization varies depending on NOM concentrations [29,30], and the value of EC_{50} also increases or decreases depending on the types of nanomaterials (e.g., TiO_2, ZnO, or Ag NPs), with and without exposure to NOM [20,31]. The difference in these toxicity values can possibly be attributable to different testing conditions and particle parameters, such as the particle size, distribution, surface coating, test medium, and ionization [16,17,22,32].

Therefore, it is essential to standardize the toxicity test methods for nanomaterials by reviewing the applicability of OECD TG 318 and developing standard testing methods. To this end, international communities such as OECD and the EU have developed TG 318 according to which nanoparticles (NPs) are dispersed in NOM; however, its applicability for ecotoxicity assessment should be further reviewed. In this study, we aimed to investigate the cause of ecotoxicity of ZnO NPs dispersed in NOM by monitoring their increased or decreased ecotoxicological tendency and identifying their dispersion stability and ionization.

Table 1. Summary of ecotoxicity of ZnO NPs in the previous studies.

Species	Exposure Period	Toxicity Values	Particle Size and Type	Exposure Media	pH	Reference
P. subcapitata	72 h	EC_{50} 42 µg/L EC_{50} 42 µg/L EC_{50} 37 µg/L	Ion ($ZnSO_4$) 50–70 nm Bulk	OECD test medium	8.0 ± 0.4	Aruoja et al., 2009 [16]
		EC_{50} 49 µg/L EC_{50} 68 µg/L EC_{50} 63 µg/L	30 nm (Dispersant) 30 nm (Powder) Bulk	EPA medium	7.5 ± 0.1	Franklin et al., 2007 [17]
Skeletonema costatum	96 h	EC_{50} 2.36 mg/L	20 nm	Artificial seawater	8.0 ± 0.1	Wong et al., 2010 [9]
Dunaliella tertiolecta	96 h	EC_{50} 2.42 mg/L	100 nm	Artificial seawater	-	Manzo et al., 2013 [32]
Daphnia magna	48 h	LC_{50} 3.2 mg/L	50–70 nm	Synthetic freshwater	7.3–7.8	Heinlaan et al., 2008 [21]
		LC_{50} 7.5 mg/L	<200 nm	Elendt M4 medium	7.7–8.4	Wiench et al., 2009 [22]
		EC_{50} 0.62 mg/L LC_{50} 1.51 mg/L	20 nm	ISO test medium	-	Zhu et al., 2009 [23]
		EC_{50} 2.6 mg/L	50–70 nm	Natural water	7.5–8.2	Blinova et al., 2010 [24]
		EC_{50} 1.9 mg/L EC_{50} 3.1 mg/L	<50 nm <100 nm	Commercial mineral water (San Benedetto®)	8.81	Santo et al., 2014 [18]
		LC_{50} 0.99 mg/L LC_{50} 1.15 mg/L LC_{50} 1.01 mg/L	43 nm 43 nm Ion ($Zn(NO_3)_2$)	ISO test medium	7.8 ± 0.2	Xiao et al., 2015 [25]
		LC_{50} 0.76 mg/L LC_{50} 1.02 mg/L LC_{50} 1.10 mg/L LC_{50} 0.89 mg/L	Ion ($ZnCl_2$) 30 nm 80–100 nm >200 nm	ASTM hard water	7.9 ± 0.3	Lopes et al., 2013 [19]
		EC_{50} 4.7 mg/L EC_{50} 2.2 mg/L	151 nm 151 nm	M7 medium M7 medium + NOM	8.2 ± 8.5	Cupi et al., 2015 [20]
		EC_{50} 0.047 mg/L EC_{50} 4.9 mg/L	151 nm 151 nm	VS EPA medium M7 medium	7 8.6	Cupi et al., 2016 [26]
Danio rerio	96 h	LC_{50} 3.97 mg/L LC_{50} 2.52 mg/L	30 nm <500 nm	Distilled water	6.9–7.3	Yu et al., 2011 [27]
		LC_{50} 1.79 mg/L LC_{50} 1.55 mg/L	20 nm 1000 nm	Distilled water	-	Zhu et al., 2008 [28]

2. Materials and Methods

2.1. Preparation of Materials

2.1.1. Nanomaterials

Given that nanomaterials are widely used in various products, we selected the metallic ZnO nanoparticles (NPs) that have a high probability of causing toxicity to aquatic organisms due to ionization in water systems. We used ZnO (CAS No. 1314−13-2, Sigma-Aldrich, St. Louis, MO, USA) with a size less than 100 nm and dispersed the NPs in 50 wt.% suspension.

ZnO NPs were dispersed in deionized (DI) water to ensure a concentration of 500 mg/L. As recommended by OECD TG 318: dispersion stability of nanomaterials in simulated environmental media [11], a concentrated solution was prepared using a probe sonicator (Ø: 13 mm) at 40 W for 10 min for dispersion. The solution was then dispersed in OECD media under TG 201: freshwater alga and cyanobacteria, growth inhibition test [33], International Organization for Standardization (ISO) media under TG 202: *daphnia* sp. acute immobilisation test [34], and dechlorinated tap water under TG 203: fish, acute toxicity test [35].

2.1.2. NOMs

The Suwannee River NOM (2R101N, International Humic Substances Society, Denver, CO, USA) was selected as the natural organic matter for dispersing the nanomaterials as recommended by OECD TG 318. NOMs are mixtures of organic substances (extracted from the U.S. Suwannee river in our study) found in soils, sediments, and natural waters.

For the NOM stock solution, the NOM powder was dispersed in DI to obtain a 500 mg/L concentration and was adjusted to pH 8 with sodium hydroxide (NaOH, 10N, RUO, BIONEER, Daejeon, Korea) for facilitating solubility, followed by 24 h of vigorous stirring. Thereafter, it was filtered with a 0.2 µm polyether sulfone (PES) syringe filter, refrigerated in a brown bottle to prevent exposure to light, and used within four weeks after being diluted with a dissolved organic carbon concentration to 10 mg/L [36].

2.2. Measurement of Dispersibility

For identifying the dispersion, aggregation, and precipitation of nanoparticles, a supernatant of a 50 mL centrifuge vial was settled, and its absorbance was measured using an UV-vis spectrometer (Evolution 260 BIO, Thermo fisher scientific, Waltham, MA, USA) in accordance with OECD TG 318. The absorbance of ZnO NPs was measured at 360 nm, followed by the dispersibility measurements.

Dispersion stability of the particles was analyzed based on the particle-size changes. The ZnO NPs were dispersed in DI water; the test media, each with a concentration of 50 mg/L, manufactured with 40 mL solutions, were obtained. TG 318 recommends that the dispersion of nanomaterials be measured at an interval of 1 h for up to 6 h; however, considering the exposure duration of each test method, such as the freshwater algae growth inhibition test, Daphnia sp. acute immobilization test, and fish acute toxicity test, the particle aggregation was analyzed for up to 96 h. For measurement, the samples were stirred and measured three times via dynamic light scattering (DLS, ELS-Z-2 analyzer, OTSUKA, Tokyo, Japan) to obtain an average of the measured size values.

2.3. Analysis of Ion Concentration

ZnO NPs were dispersed in DI water, OECD medium, ISO medium, and dechlorinated tap water at 50 mg/L concentrations for measuring dissolved Zn^{2+} concentrations. The NP-dispersed solutions were then analyzed with and without NOM. OECD medium, ISO medium, and dechlorinated tap water were used as the test mediums following OECD TGs 201, 202, and 203 for each toxicity test at different exposure times of 72, 48, and 96 h, respectively, for each test medium. At 24 h intervals, the ionizations in the OECD medium, ISO medium, and dechlorinated tap water were measured three,

four, and five times, respectively. The ionization in DI water was measured five times for an exposure time of 96 h. Ten mL of each solution was injected into ultra-4 centrifugal tubes (Amicon Ultra−15, Centrifugal Filters Ultracel-3K/30K, Merck Millipore Ltd., Burlington, MA, USA) containing three filters with pore sizes of 3 and 30 kDa and centrifuged at 5000 rpm for 30 min. Most of the solutions that passed through the 3 kDa filter possessed free Zn^{2+}, whereas those that passed through the 30 kDa filter contained free Zn^{2+} and conjugated ions. For analyzing the ion concentrations of ZnO NPs, an appropriate acid treatment was required. We collected 1 mL of the centrifuged solutions that passed through the 3 or 30 kDa filter, then added 1 mL nitric acid (HNO_3, 69%, GR, WAKO, Tokyo, Japan), and mixed them gently. The mixed solution was allowed to settle for 15 min [37,38] and was then diluted with 8 mL of DI water.

For analyzing the ion concentrations of MNs, a proper acid treatment is required to ionize the particles. One mL of centrifuged supernatant solution was collected and acidified in a 15 mL tube. ZnO NPs were pretreated by stirring with 1 mL of nitric acid, settling for 15 min [37,38], and dispersing with 8 mL of DI water.

ZnO NPs were analyzed using an ICP/MS (Agilent 9700 × quadrupole, Agilent Technologies, Santa Clara, CA, USA) and the data were processed using the Mass hunter 7.0 software in spectrum mode. An element measurement was conducted using the spectrometer parameters. The analysis conditions were 1550 W of output power from a RF generator (radio frequency generator) and a 1 L/min nebulizer gas flow rate; these are important parameters for sensitivity interrupting oxidation and double-charge ion formation. With a sample-uptake flow rate of 1 mL/min and sweeps-counting signal pulses of 100, the samples were measured with three replicates.

2.4. Characterization of ZnO Nanoparticles

The dispersed ZnO NPs were measured via transmission electron microscopy (TEM) and Fourier-transform infrared (FT-IR) spectroscopy. We prepared 40 mL of dispersed ZnO NPs with 50 mg/L in DI water with and without 10 mg/L of NOM according to Sections 2.1.1 and 2.1.2 and subsequently reacted for 96 h. For measuring the NP size via TEM (H7650, Hitachi, Tokyo, Japan), the dispersed solution was placed on a copper-coated grid and left for more than 6 h at 24 °C to dry completely. The sizes of ZnO NPs were measured, of which 20 were measured via image J (distributed by NIH) [39]. To measure the surface reactivity via FT-IR (TENSOR 27, Bruker, Billerica, MA, USA), the solution was centrifuged at 10,000 rpm (Hanil, supra 22k, Seoul, Korea) for 10 min and dispersed with DI water into three replicates. The measurement range of FT-IR was between 400 and 4000 cm^{-1}.

The physicochemical properties of nanomaterials, surface charges (DLS, ELS-Z-2 analyzer, OTSUKA, Tokyo, Japan), and pH (Orion versa star advanced electrochemistry meter, Thermo fisher scientific, Waltham, MA, USA) were measured in DI water and/or each test media, based on the presence/absence of NOM.

2.5. Ecotoxicity Study

2.5.1. Test Media of Nanomaterials Dispersed with NOM

In order to determine the toxic effect and the improved dispersibility of nanomaterials with NOM, ecotoxicity tests (TG 201 [33], 202 [34], 203 [35]) were conducted with the adaption of OECD TG 318. OECD TG 318 recommends that NOM should be used for the improved dispersibility of nanomaterials. Therefore, we conducted ecotoxicity tests with/without NOM.

The ecotoxicity test media were prepared according to each test method. OECD media were used for the algae growth inhibition tests, ISO media for water flea acute toxicity tests, and dechlorinated tap water for fish acute toxicity tests.

We prepared 100 mg/L of NOM stock solution by adding NOM to each medium and stirring for more than 24 h (pH 8.0, 0.01 g NOM/100 mL OECD media).

Followed by stirring, the solution was dispersed according to the method explained in Section 2.1.2, resulting in ZnO dispersions in the ecotoxicity test mediums.

2.5.2. Conditions for Algae Growth Inhibition Tests

The algae growth inhibition test was conducted according to OECD TG 201, and the alga (*Pseudokirchneriella subcapitata*) used was sub-cultured once per week under the conditions of 23 ± 2 °C, 24 h-light (6000 lux), and 100 rpm (Vision Scientific Co., Ltd., Daejeon, Korea). For quality control, the growth inhibition test was initially conducted with a standard material, potassium dichromate ($K_2Cr_2O_7$, CAS No. 7778−50-9, Sigma-Aldrich, St. Louis, MO, USA) with three replicates.

The control group test was performed using OECD media, with and without NOM. Exposure concentrations were set to 53.9, 70.0, 91.0, 118.4, 153.9, 200, and 260 µg/L (common ratio 1.3) in the absence of NOM and 25, 50, 100, 200, and 400 g/L (common ratio 2.0) in the presence of NOM. The test solution was inoculated with algae at a concentration of 5×10^3 to 1×10^4 cells/mL in a triangular flask with a capacity of 100 mL. During the exposure period, the test was performed in a static condition under which the test solution was not replaced, and the rows and columns of flasks in the shaking incubator were moved sequentially at intervals of 24 h for uniform illumination. Samples were collected from all the flasks of the control and treatment groups after 24, 48, and 72 h exposure periods, the number of algae were counted using a Vi-CELL XR cell counter (Beckman Coulter, Brea, CA, USA), and a microscope was used to calculate the inhibition rate of the average specific growth rate and derive EC_{50}.

2.5.3. Conditions for Water Flea Acute Toxicity Test

The water flea acute toxicity test was performed in accordance with OECD TG 202 using *Daphnia magna* aged less than 24 h, and the immobilized organisms were observed after 24 and 48 h of exposure. In the control group test with ISO media in the presence and absence of NOM, the exposure concentrations were set to 0.625, 1.25, 2.5, 5, and 10 mg/L (common ratio 2.0) without NOM, and 1.91, 3.43, 6.17, 11.11, and 20 mg/L (common ratio 1.8) with NOM. Five water fleas were exposed to each concentration with four replicates, totaling 20 water fleas for each concentration. The exposure conditions were set at 20 ± 1 °C with a photoperiod of 16 h (light)/8 h (darkness), and no food was supplied during the exposure period.

2.5.4. Conditions for Fish Acute Toxicity Test

The fish acute toxicity test was performed in accordance with OECD TG 203, and the test species was *Oryzias latipes* (Japanese medaka) with an average length of 1.4 ± 0.1 cm and an average weight of 23.5 ± 8.1 mg, taking into account the recently revised test guideline. The control group test was conducted in dechlorinated tap water with and without NOM, and the exposure concentrations were 26.9, 35, 45.5, 59.2, 76.9, and 100 mg/L (common ratio 1.3). Eight fish were exposed to each concentration in the 1 L beakers for 96 h (0.8 g wet weight fish/L), and the lethal and abnormal behavior was observed every 24 h. The exposure conditions were set at 25 ± 2 °C with a photoperiod of 16 h (light)/8 h (darkness), and no feed was supplied during the exposure period. In this project (NIER−19-2), the animal experiments were performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Environmental Research (NIER), Republic of Korea.

2.5.5. Statistical Analysis

We calculated the rates of algae growth inhibition, water flea immobility, and fish acute toxicity values using the Probit Analysis Program (USDA, U.S. Department of Agriculture, Washington, DC, USA) [40].

3. Results and Discussion

3.1. Determination of Dispersibility

ZnO showed similar absorbance ratios of 0 to 96 h because both samples, without NOM (96 h, Ar = 0.92) and with NOM (96 h, Ar = 0.95), were fairly dispersed in DI water (Figure 1a). The specific gravity of ZnO was lower than that of water ranging from 0.3 to 0.8; therefore, precipitation did not occur, due to the specific gravity up to 96 h [41]. The addition of NOM caused a slight increase in the dispersion of nanomaterials.

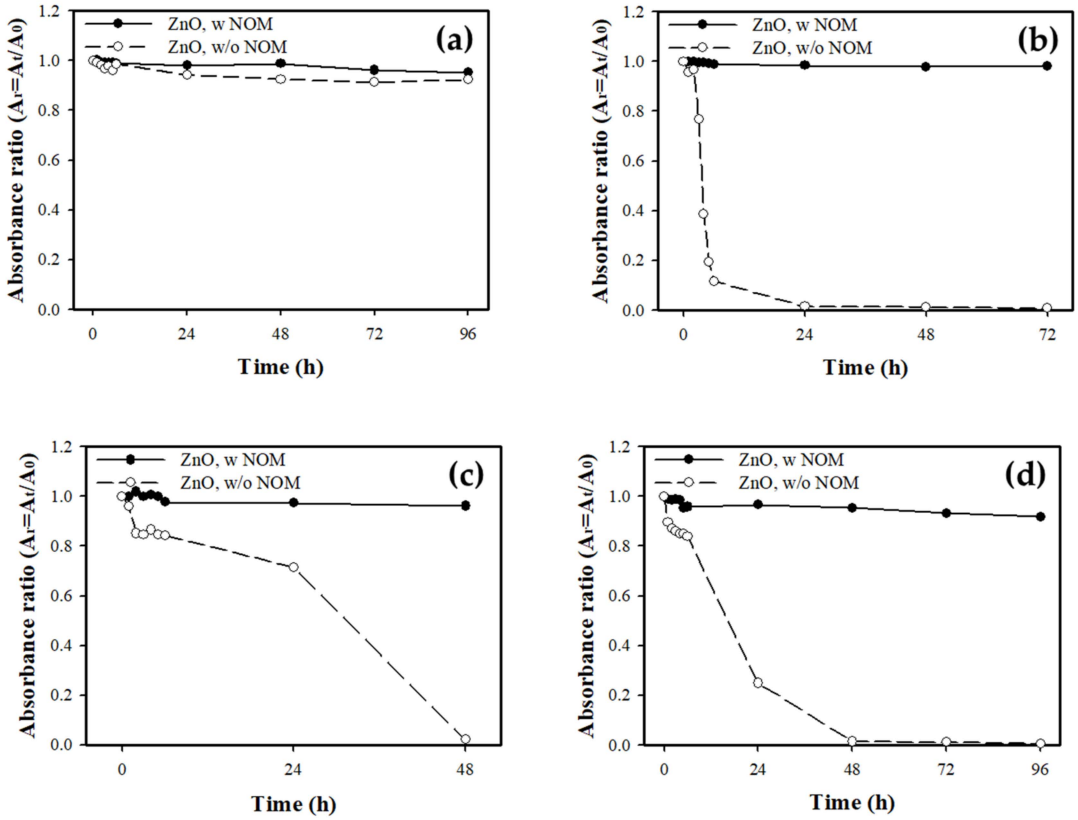

Figure 1. Sedimentation kinetics for zinc oxide nanoparticles (ZnO NPs) with/without natural organic matter (NOM) under deionized water (DI) water (**a**), Organization for Economic Co-operation and Development (OECD) medium (**b**), International Organization for Standardization (ISO) medium (**c**), and dechlorinated tap water (**d**).

In the case of the ZnO dispersed in OECD algae growth media (72 h, Ar = 0), the media without NOM were precipitated owing to aggregation; however, the media with NOM (72 h, Ar = 0.98) were well-dispersed without aggregation (Figure 1b).

In the case of the ZnO dispersed in ISO water flea test media, the media without NOM (48 h, Ar = 0.02) were aggregated only to be precipitated, while those with NOM (48 h, Ar = 0.96) were well-dispersed (Figure 1c). Both the samples with and without NOM were aggregated, but the NOM-added samples were well-dispersed.

In the case of the ZnO dispersed in fish test medium, the media without NOM were aggregated, resulting in precipitation, but the media with NOM (96 h, Ar = 0.92) did not show precipitation and were well-dispersed (Figure 1d). The significant improvement in the stability of ZnO in the presence of NOM can be attributed to steric hindrance as reported by many previous studies [29,30,42].

3.2. Measurement of Properties

3.2.1. Particle-Size Determination via TEM

As shown in Figure 2, the particle sizes of ZnO NPs dispersed with and without NOM were analyzed using TEM images. The average size of ZnO NPs was 36.48 ± 6.61 nm (n = 20), and that of ZnO NPs dispersed with NOM was 36.10 ± 9.13 nm (n = 20). However, it is difficult to identify the increase in size resulting from the NOM dispersion using the TEM images. In addition, it was confirmed that the addition of NOM led to the reduced sharpness of the particles, and a thin film of about 2 nm was formed on the particle surface.

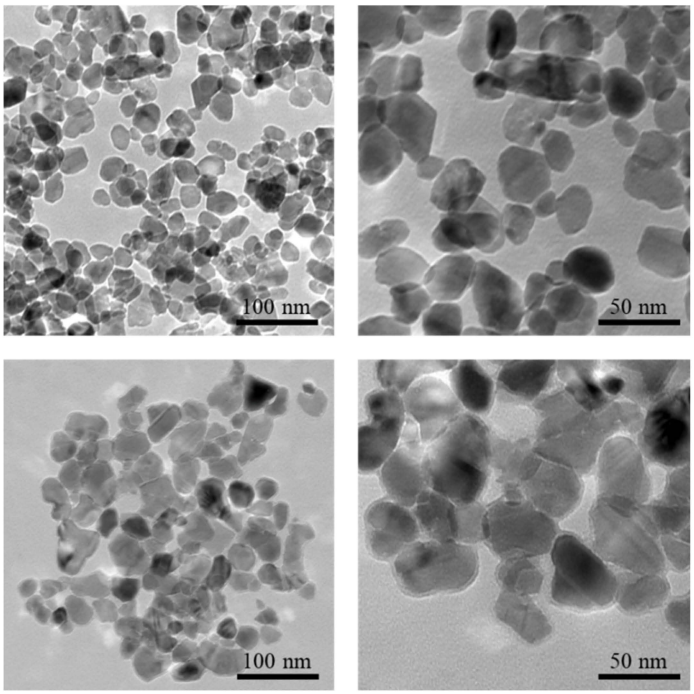

Figure 2. Transmission electron microscopy (TEM) image of ZnO. Top (without NOM), down (with NOM).

3.2.2. Determination of Surface Charge of Nanoparticles in Test Medium

It is shown in Figure 3 that the surface charge of ZnO changed from positive to negative with the addition of NOM, and the overall increase in the absolute value indicates an excellent dispersion stability. The NOM with negative charge increasingly tended to coat the surface of ZnO with positive charge, improving the dispersion stability [43], and, generally, remained stable above the absolute value of 30 mV [44]. pH was found to range from 7.53 to 7.99, with and without NOMs in the OECD medium, ISO medium, and dechlorinated tap water test medium, suggesting that the range was acceptable for ecotoxicity tests without further pH adjustment.

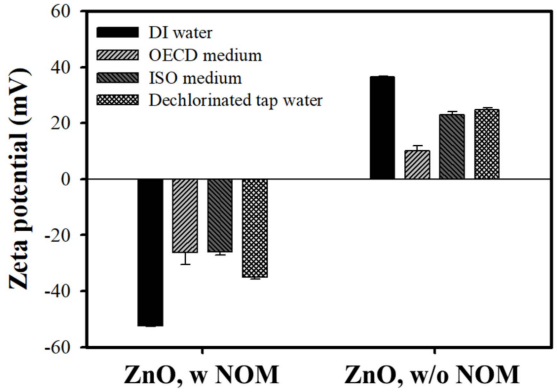

Figure 3. Zeta potential of ZnO NPs with/without NOM. Values on the graph represent mean ± standard deviation.

3.2.3. Measurement of Surface Reactor

For analyzing the surface reaction of ZnO NPs, an FT-IR measurement was conducted at wavelengths of 400–4000 cm^{-1} in an aqueous solution. The fundamental frequency and a high-value absorption band of 422.39–553.53 cm^{-1} correspond to the skeletal vibrations of ZnO as a fingerprint region, which is equivalent to 454.75 and 442.60 cm^{-1}, with and without NOM, respectively (Figure 4). The absorption band of 1250–1050 cm^{-1} is a stretching vibration of carbon and nitrogen (C–N) in the local amines, while that of 1340.38–1553.08 cm^{-1} is an asymmetric and symmetric stretching vibration of carbonyl function groups C=O and C–O, and that of 2800–3000 cm^{-1} is for the CH_2 and CH_3 of methyl groups. The findings are attributed to the presence of zinc acetate dihydrate used in manufacturing ZnO [45], demonstrating the same results regardless of the presence of NOM. In addition, the samples with only NOM showed an absorbance of 3208.41 cm^{-1} that corresponds to the stretching vibrations of oxygen and hydrogen in the alcohol and phenyl radicals and to the O-H and H-bonded zone, with a range of 3500–3200 cm^{-1}. These results show that NOM was present on the surface of ZnO NPs [46].

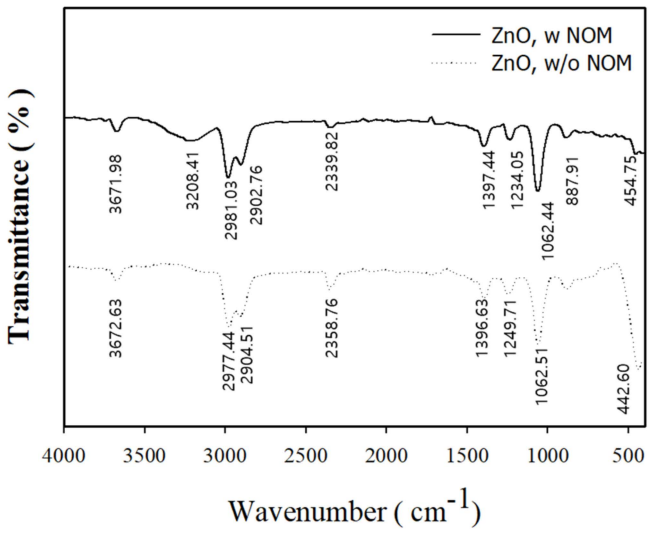

Figure 4. Fourier-transform infrared spectra of ZnO NPs with/without NOM.

3.2.4. Determination of Particle Size and Distribution in Test Medium

The absolute value of the surface charge of ZnO dispersed in DI water was 52.35 mV with NOM and 36.47 mV without NOM, indicating that the absolute value above 30 mV was high enough to be completely dispersed [44] (Figure 3).

The average particle size analyzed by DLS was 36.5 nm, suggesting a stable dispersion. Aggregation was not detected even after 96 h of exposure in the state of being dispersed in DI water; the particle size was maintained at 46.4 nm. Further, the size of ZnO with NOM was maintained at 44.4 nm without aggregation after 96 h of exposure (Figure 5a).

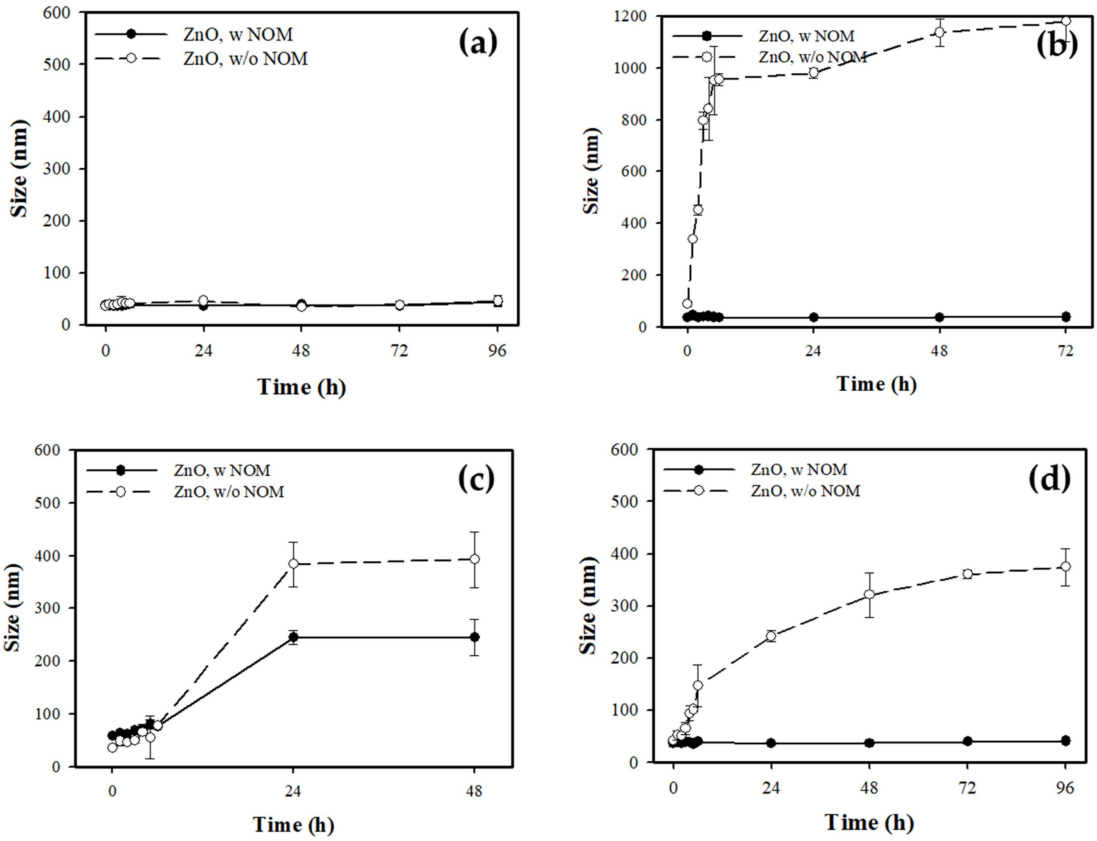

Figure 5. Hydrodynamic diameter of ZnO NPs with/without NOM in DI water (**a**), OECD medium (**b**), ISO medium (**c**), and dechlorinated tap water (**d**).

The surface charge of the ZnO dispersed in OECD algae growth test medium was 0.02 mV, indicating a value of approximately 0; this was a condition that is prone to aggregation. ZnO with NOM showed a high absolute value of 26.34 mV, suggesting a good dispersion (Figure 3). The particle sizes of ZnO, with and without NOM, significantly decreased to 36.7 nm from 1178.7 nm after 72 h of exposure; this is attributable to excellent dispersion (Figure 5b).

The absolute surface charge of the ZnO dispersed in ISO water flea test medium was 23.00 mV, similar to the absolute surface charge of ZnO in the presence of NOM, which was 26.12 mV (Figure 3). However, the particle size of 392.63 nm after 48 h of exposure decreased to 245.03 nm with a reduced aggregation due to the addition of NOM (Figure 5c). Despite a similar surface charge, the addition of NOM improved the dispersion because the NOM coated on the surface of nanomaterials resulted in steric hindrance that made it difficult to access the spaces between the nanomaterials owing to the size of the NOM molecules [29,30,42].

The absolute value of surface charge of the ZnO in the fish test medium was 24.84 mV that slightly increased to 35.11 mV after the addition of NOM, suggesting that the presence of NOM would further facilitate dispersion (Figure 3). The ZnO particle size measured by ELS was 374.37 nm but decreased to 40.87 nm with the addition of NOM, indicating excellent dispersion (Figure 5d).

The ionic strength increased in the ecotoxicity test medium compared to the DI water and, therefore, the diffused double-layer thickness of ZnO NPs was reduced. This led to a decreased zeta

potential (Figure 3), thereby reducing the inter-particle repulsive force that reduces the aggregation rate of ZnO NPs. It is reported that the dispersion and aggregation of ZnO NPs are strongly affected by pH changes; however, in this study, the pH changes were slight in the range of 7.53 to 7.99.

3.2.5. Analysis of Ionic Effect

With aqueous complexation reactions with metal ions, NOM could not permeate through the 3 kDa filter, although the 30 kDa filter was permeable, thus facilitating NOM separation. All samples were pretreated as described in Section 2.3 and diluted 10 times. To express the measured concentration correctly, the diluted concentration was multiplied 10 times. Therefore, using two types of ultra-4-centrifugal filters, 3 and 30 kDa, we could determine whether aqueous complexes were formed [47,48].

As seen in Figure 6, the presence of NOMs resulted in different ionic concentrations of the solutions that passed through the 3 and 30 kDa filters at 96 h for DI water, 72 h for OECD medium, 48 h for ISO medium, and 96 h for dechlorinated tap water. The ionic concentrations of the test mediums were 2.10, 0.28, 0.12, and 0.61 mg/L in the presence of NOM, whereas they were 0.51, 0.11, 0.06, and 0.30 mg/L in the absence of NOM, respectively. In other words, all the test mediums showed higher ion concentrations when NOM was present, suggesting that NOM formed complexes with Zn^{2+}. As complex formation with NOM depends on the types of ions (monovalent and divalent), with more significant effects from divalent cations, the formation of different complexes prepared in different media depends on the ion species.

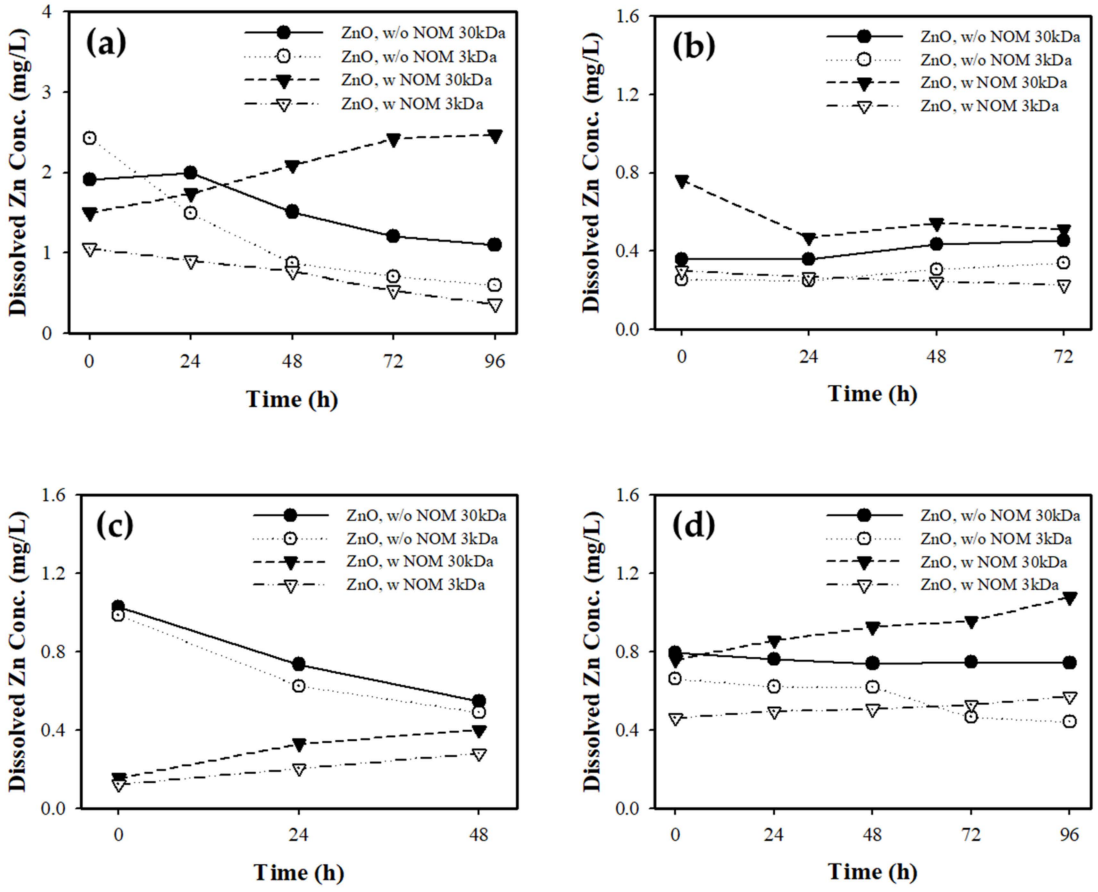

Figure 6. Dissolution of ZnO NPs with/without NOM in DI water (**a**), OECD medium (**b**), ISO medium (**c**), and dechlorinated tap water (**d**).

The ZnO NPs dispersed in DI water and the test medium was detected with a low ion concentration of less than 3 mg/L in all samples [43,49]. The degree of ionization is known to increase with the effects of pH, ion concentration, and temperature, and, in particular, the pH effect is significant [37]. As the pH range of the ecotoxicity test medium with ZnO NPs did not significantly change, ranging from 7.53 to 7.99 (Table S1), we concluded that the effect of pH on the degree of ionization was not significant. The concentration of ZnO ions in DI water was higher than the concentrations in other mediums due to a low pH of 5.78 in DI water, resulting in increased solubility [14]. There is little change in the ionization over time, and the change in ionic amount during the exposure period under the OECD test method indicated a low value regardless of the presence of NOM (Figure 6). The concentration differences between the solutions with and without NOM that passed through the 30 kDa filter were not significant. When the solutions from the last samples of the OECD and ISO mediums passed through the 30 kDa filter, their ion concentrations with NOM were 0.51 mg/L for the OECD medium and 0.40 mg/L for the ISO medium, whereas for those without NOM, they were 0.45 mg/L for the OECD medium and 0.55 mg/L for the ISO medium. As ZnO demonstrated few ionic changes with NOM, we inferred that the ionic effect on toxicity was also not significant.

As the NOM forms aqueous complexes, a solution obtained from filtering using the 30 kDa filter paper was expected to exhibit a higher concentration than the 3 kDa filter paper; however, the ionized concentration was low and could not be verified. With slight ionic changes, ZnO NPs is not expected to exhibit a large ionic effect during the toxicological evaluation.

3.3. Ecotoxicological Effect of NOM on ZnO

The sensitivity test was conducted with potassium dichromate ($K_2Cr_2O_7$), a reference material, to verify the reliability of the growth inhibition test with *Pseudokirchneriella subcapitata*. The results showed that the growth rate (μ) of algae decreased as the concentration of the reference material increased, indicating that the EC_{50} value, the average coefficient of variation (C.V.) of the specific growth rate, and the C.V. of the average specific growth rate fell to the acceptable limits of 137.7 ± 2.0 μg/L, 30.9% (within 35% of critical limit), and 5.6% (within 7%), respectively.

In the algae growth inhibition test of ZnO NPs, based on the EC_{50} values with and without NOM, it is shown that both the control and positive control groups with NOM have negligible difference in the growth rates compared to the initial inoculation volume after 72 h of exposure; this suggests that NOM did not have any direct impact on the algae growth (Figure S1). In addition, the average C.V. of the specific growth rate due to the control group's interval indicated an appropriate range (Figure S1).

The EC_{50} (72 h) of ZnO NPs was 137.7 ± 2.0 μg/L, and the EC_{50} (72 h) with NOM was 397.6 ± 12.3 μg/L, which was a threefold decrease in algae growth inhibition, resulting in decreased toxicity effects (Figure 7).

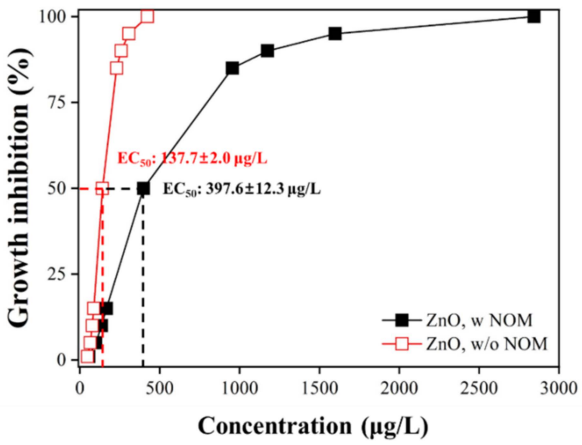

Figure 7. Algae growth inhibition by ZnO NPs with/without NOM.

In the sensitivity test of water fleas, the 24 h-immobilization rate using potassium dichromate ($K_2Cr_2O_7$) as a reference material increased to 1.41 mg/L (EC_{50}) with the concentrations falling into an acceptable range of 0.6 to 2.1 mg/L, suggesting that it was suitable to conduct the acute toxicity test. In the water flea acute toxicity test of ZnO NPs, immobilized individuals were observed in all concentrations greater than 1.25 mg/L, particularly at immobilization rates of 40%, 80%, and 100% in concentrations of 1.25, 2.5, and 5 mg/L, respectively, with 48 h-EC_{50} at 1.55 mg/L (Figure 8). The EC_{50} of ZnO NPs dispersed with NOM was 12.92 mg/L, which is an 8.3 fold increase, indicating a decrease in toxicity owing to the presence of NOM (Figure 8). In the study conducted by Cupi et al., when *Daphnia magna* were exposed to ZnO NPs for 48 h, the opposite trend was observed as their toxicity increased [20] to 1.5 mg/L (EC_{50}) with addition of NOM from a value of 4.7 mg/L (EC_{50}) without NOM. We attributed this trend to the different surface property of ZnO NPs that caused increased dissolution in the presence of NOM, resulting in increased toxicities as well. In the case of Ag NPs, similar tendencies were observed as toxicity decreased to a value greater than 100 mg/L in the presence of NOM from 41.3 mg/L (EC_{50}). Based on the findings, it was confirmed that NOM did not directly affect toxicity in water fleas, because no immobilization was observed in the control and positive control groups.

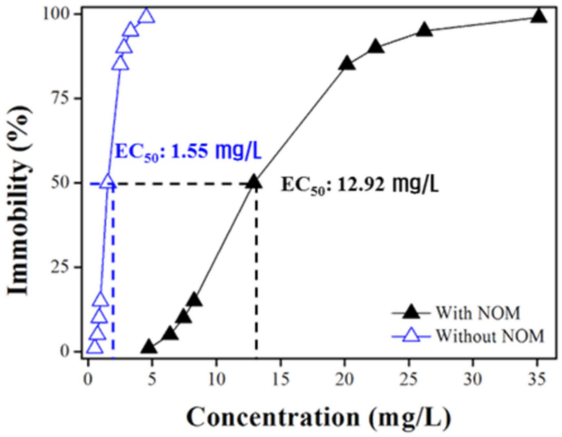

Figure 8. *Daphnia magna* immobility by ZnO NPs with/without NOM.

In the acute toxicity test of ZnO NPs with *Oryzias latipes*, no deaths were detected at the lowest concentration (26.9 mg/L) in the control group without NOM. However, mortality rates were observed as 10%, 85%, and 95% at 38.4, 58.2, and 61.8 mg/L, respectively, indicating that 96 h-LC_{50} was 47.31 mg/L (Figure 9). However, in the presence of NOM, no mortalities were seen at any concentration level with LC_{50} > 100 mg/L, suggesting that the toxicity was significantly alleviated in the presence of NOM.

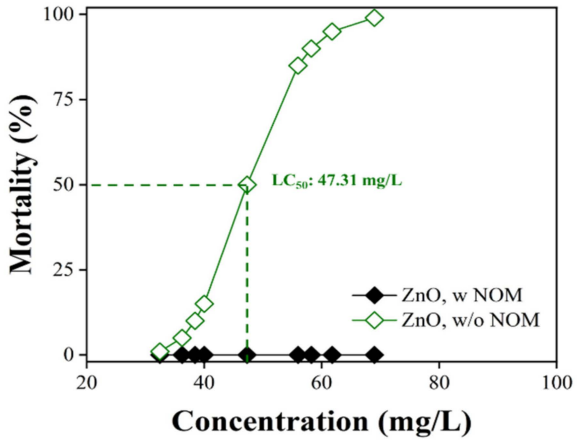

Figure 9. *Oryzias latipes* mortality by ZnO NPs with/without NOM.

Based on the above results, it was confirmed that the toxicity values (EC_{50}, LC_{50}) for algae, water fleas, and fish was lowered in the presence of NOM. Note that the control tests showed that no direct toxic effects were observed from NOM for all cases. Previous studies reported that Zn ions are toxic, but our dissolution test results in Section 3.2.5 revealed that ZnO dissolution is negligible regardless of the presence of NOM, suggesting that ion species distribution is not the reason. Hence, we turned our attention to the stability (i.e., dispersibility) of ZnO without versus with NOM. The presence of NOM increased the dispersibility of ZnO and reduced the toxicity level for all cases, which indicates the strong relationship between ZnO dispersibility and toxicity level. At present, the exact toxicological mechanisms are not clear, and it requires further study.

To identify the causes of reduced Zn toxicity with NOM, we studied relevant literature on ionization tendencies. We observed that, besides ionization effects, several critical parameters influenced Zn toxicity, including the reduction in oxidative stress caused by reactions between NOM and free radicals Reactive Oxygen Species (ROS) and the reduction in toxicity through passivation of particle surfaces [14,15,50–52]. Based on these findings, further investigation is required to clarify the mechanism for reduced toxicity in the presence of NOM.

4. Conclusions

In this study, we applied TG 318 as the method of dispersion of nanomaterials to our ecotoxicity test, and the following conclusions were confirmed:

ZnO was fairly dispersed in DI water without aggregation regardless of the presence of NOM, whereas excellent stability was only observed with NOM in the ecological toxicity media. For the ecological toxicity test, the addition of NOM in the test medium of algae, water fleas, and fish resulted in less aggregation and confirmed the effect of dispersion.

In the FT-IR analysis of NOM-coated ZnO, alcohol and phenyl radical bonds were confirmed in the range of 3500–3200 cm^{-1}. In addition, the surface charge indicated by the zeta potential was changed from positive to negative owing to the presence of NOM; therefore, the absolute value was increased. As a result, the analysis of particle sizes showed that the particles were small and well-dispersed in the ecotoxicity test medium.

During the exposure with or without NOM, the ionic concentrations in each ecotoxicity test medium hardly changed to a value below 2 mg/L, implying that ionization would have had no effect on the toxicological variations.

It is regarded that NOM has a dispersion effect on nanomaterials and is an appropriate dispersant because no toxic effects are seen on algae, water fleas, and fish. However, further investigation is required to establish a standard test method for the evaluation of toxicities of nanomaterials.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/18/6431/s1, Figure S1: Comparison of algae growth inhibition by ZnO depending on the NOM, Table S1: Zeta potential and pH changes of test medium with ZnO with/without NOM.

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