Rapid In Situ Biomonitoring of Subsoil Contamination by Applying an Algae-Soaked Disc Seeding Assay

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Abstract: Various pollutants are pervasive in soil environments due to human activities, thereby damaging soil ecosystems. In this study, extension of a previously developed algae-soaked disc seeding assay for periodic evaluation of subsoil contamination over time was described. The assay can be used in different contamination configurations of silver nanoparticles in combination with examination of cell morphology, esterase activity, oxidative stress, and membrane permeability. In addition, we periodically attempted to repeat the algae-soaked disc seeding assay every three weeks. We evaluated applicability of this algae-soaked disc seeding assay using alga Chlamydomonas reinhardtii exposed to heterogeneous silver nanoparticle-contaminated soils. The results demonstrated that this assay is applicable for monitoring a change of subsoil contamination by periodic evaluation over time. The developed assay was identified as a periodically rapid in situ biomonitoring technique to measure subsoil contamination over time.

Keywords: biomonitoring; Chlamydomonas reinhardtii; ecotoxicity; nanoparticle; soil alga; subsoil contamination

1. Introduction

Monitoring soil pollution is very important for protection of other environmental media because there is potential behavior of soil pollutants via hydrological transport or volatilization to other environmental media (e.g., groundwater, surface water, sediments, or air) [1]. For monitoring soil pollution effectively, it is necessary to consider an integrated approach with practical bioavailability by biomonitoring and measured chemicals by analytic chemistry [2]. The soil quality of a target site is investigated by biological indicators as well as physicochemical indicators, such as plants [3,4], earthworms [5,6], enzyme activity [7], and soil algae [6,8]. However, these chemistry and toxicity data of the target soil resulted from temporarily short-term experiments. Only in situ soil dehydrogenase activity in soils treated with tebuconazole or azoxystrobin and amended with spent mushroom substrate was investigated in a periodic long-term experiment, over 355 or 378 days [9,10]. This traced the change of in situ soil dehydrogenase activity induced from tebuconazole or azoxystrobin over time (0, 36, 92, 124, 355 days or 0, 28, 84, 181, 378 days). Therefore, it is necessary to conduct periodic subsoil contamination monitoring for prevention of environmental effects resulting from unpredictable subsoil contamination (e.g., oil leaks, radioactive effluents, or illegal disposal).

Soil algae are a key food source for mesofauna and microfauna [11]. In addition, they synthesize organic matter and form mucilaginous sheaths that are essential to maintain soil fertility and aggregation [11]. In a previous study [12], we developed an algae-soaked disc seeding assay as a rapid in situ assessment for predicting soil quality and presented decrease of chlorophyll fluorescence of the alga Chlamydomonas reinhardtii after exposure to contaminated, remediated, and amended farm and paddy soils in the field using the algae-soaked disc seeding assay. The results of that study identified that the algae-soaked disc seeding assay is convenient for rapidly assessing in situ soil contamination. However,
Nam et al. [12] only assessed chlorophyll fluorescence of *C. reinhardtii* in solely heterogeneous soil (30 cm of clean topsoil plus 70 cm of target subsoil) collected from the field at 2 days. Soil algae, as terrestrial organisms lived on and beneath the soil surface [11], can be a terrestrial test species as a periodic toxicity indicator in the long-term experiment. In this study, we extended our experiment to assess its applicability to periodic evaluation for subsoil contamination under different contamination configurations of heterogeneous soils, together with examination of cell morphology, esterase activity, oxidative stress, and membrane permeability using an algae-soaked disc seeding assay. Silver nanoparticles (AgNPs) were used as a target chemical because of their broad use in our life [13,14] and the ecotoxicity of AgNPs on soil organisms: plants, isopods, earthworms, soil algae, and exoenzymes [15–19]. *C. reinhardtii*, which is conventionally living in freshwater and soil, has been used as a biological indicator [6,12]. To the best of our knowledge, this is the first study to assess applicability of an algae-soaked disc seeding assay considering periodic evaluation of subsoil contamination under different contamination configurations of heterogeneous soils, as part of an efficient algae-soaked disc seeding assay for rapid in situ biomonitoring of subsoil contamination over time.

2. Materials and Methods

2.1. Test Species and Pre-Culture

*C. reinhardtii* (wild type; UTEX # 2244) was purchased from the University of Texas, Austin, USA (UTEX, https://utex.org, accessed on 20 June 2020). Cultures were incubated in a Tris-acetate-phosphate medium. The cells were incubated at 24 ± 2 °C in 250 mL borosilicate glass flasks fitted with air-permeable stoppers and shaken at 100 r/min, under 16:8 h (light:dark) illumination cycles provided by cool-white fluorescent lamps (approximately 54 µmol photons/m²s). Aliquots of the algal samples were used during the exponential growth phase (OD<sub>660</sub> 2.0).

2.2. Test Chemical

Powdered silver nanoparticles (AgNPs; <100 nm; 99.5% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA), with 0.2% polyvinylpyrrolidone as a dispersant. The morphology and surface area of the AgNPs were observed using a field emission transmission electron microscope (FE-TEM; Tecnai G2 F30 ST, FEI, Dawson Creek Drive Hillsboro, OR, USA; 300 kV) and a surface area analyzer (Microtrac, York, PA, USA). AgNPs had an irregular morphology and an average of the surface area of 1.8498 ± 0.0191 m²/g, as described in Figure 1a. The distribution and concentration of AgNPs in soil with a dry weight of 100 mg AgNPs/kg were observed using an analytical high-resolution scanning electron microscope (HR-SEM; SUPRA 55VP; Carl Zeiss, Oberkochen, Germany) with an energy-dispersive X-ray spectroscopy detector (EDX; XFlash Detector 5010; Bruker, Billerica, MA, USA). The surface of the 100 mg AgNPs/kg (dry weight) soil displayed electron dense spots for Ag, according to the SEM image and EDX spectra in Figure 1b–h.
Figure 1. Field-emission transmission electronic micrograph (FE-TEM) of silver nanoparticles (AgNPs) (a) and high-resolution scanning electron microscopy (HR-SEM) images of AgNPs in 100 mg AgNPs/kg (dry weight) test soil. Adsorption of AgNPs on the soil surface was assessed with an energy-dispersive X-ray spectroscopy (EDX). (b–e) are HR-SEM images and (f–h) indicate EDX spectra of an electron dense spot. The red circles show the presence of AgNPs on the soil surface and blue circles show absence of AgNPs in the normal soil.

2.3. Test Soil

A natural loamy sand, LUFA 2.2 soil (LUFA-Speyer, Sp 2121, Speyer, Germany), was used as the test soil. The physicochemical properties of LUFA 2.2 soil were as follows: a water-holding capacity of 0.54 mL/g, an organic matter of 3.39%, a pH of 5.6, a total nitrogen of 375 mg/kg, an available phosphate of 24 mg/kg, a calcium of 9.43 Cmol+/kg, a potassium of 0.18 Cmol+/kg, and a magnesium of 0.4 Cmol+/kg. AgNPs were spiked in LUFA soil that was autoclaved for 15 min at 121 °C, at the nominal concentration of 100 mg AgNPs/kg dry weight, and then thoroughly mixed with a roller at 40 r/min for 24 h.

2.4. Experimental Design to Evaluate the Periodic Influence of Silver Nanoparticles (AgNPs) Toxicity over Time

Four contamination configurations of heterogenous soils were prepared in vials, as described in Figure 2: control, 1st, 2nd, 3rd, and 4th layer-treated groups. Two grams of AgNP-free soil was put in a glass vial (height 40 mm, diameter 18 mm) and saturated with an aliquot of autoclaved deionized water. This process was repeated four times to obtain a final 8 g of soil with 90% of the maximum water holding capacity in each vial. The AgNP-free soil was then substituted with 100 mg AgNPs/kg dry weight soil for each exposure scenario. According to the modified method reported in Nam et al. [12,20], we dipped a disc made of pure cellulose fibers (diameter 10 mm, thickness 1.1 mm; Advantec, KS, USA) into the algal suspension and then placed it in each vial, pressing it adhered to the soil surface. At the beginning of the experiment, the vials were incubated for six days under the same conditions as those used to sub-culture the algae, except that the vials were not shaken. After three weeks, 0.3 mL of deionized water was added to each vial and the disc containing the algal suspension was placed on top of the latter. Once again, the vials were incubated for six days under the same conditions as those used to sub-culture the
algae, except the vials were not shaken. The process was repeated after 3, 6, 9, 12, 15, 18, and 21 weeks. The entire experiment lasted approximately six months and was performed with six vials per exposure scenario to obtain three replicates. The first three vials were used to analyze biomass and cell morphology; the others were used to analyze esterase activity, oxidative stress, and membrane permeability.

To analyze the biomass of the *C. reinhardtii*, algal chlorophyll a was extracted after mixing 0.05 mL algal suspension and 0.2 mL ethanol in dark conditions (3 h, 24 ± 2 °C, 100 r/min). The fluorescence of chlorophyll a was measured using a fluorescence microplate reader (Gemini Molecular Devices, Orleans Drive Sunnyvale, CA, USA), with 420 nm as the excitation wavelength and 671 nm as the emission wavelength [21].

To analyze the cell morphology of the *C. reinhardtii*, algal suspensions were measured using a flow cytometer (FACScalibur, BD Biosciences, Franklin Lakes, NJ, USA), with forward-scattered light (FSC) and side-scattered light (SSC). FSC and SSC parameters indicate cell size and granularity, respectively. The gating region in the dot plots was adjusted to divide algal populations and the background (e.g., soil fine particles), and data were acquired from 10,000 events in the region. The geometric means of the FSC and SSC intensities were analyzed using FlowJo V10 (FlowJo LLC, Ashland, OR, USA).

To analyze esterase activity, oxidative stress, and membrane permeability of the *C. reinhardtii*, the algal suspensions were centrifuged at 2500 r/min for 5 min and re-suspended in 2.7 mL of Bold’s basal medium. Algae were stained with fluorescent dye calcein-acetoxyethyl ester for esterase activity, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) for oxidative stress or fluorescein diacetate for membrane permeability, according to Nam et al. [20], Brussaard et al. [22], and Michels et al. [23]. FL1 (500–560 nm band pass filter, excitation at 488 nm blue laser, 15 mW, argon ion laser) was used to measure esterase activity, oxidative stress, and membrane permeability. In addition, unstained algae were measured using FL1 to verify autofluorescence. The gating region, number of events, statistics of FL1 intensity, and FlowJo V10 were determined by cell morphology analysis.
2.6. Statistical Analysis

The percentage of biomass, cell morphology, esterase activity, oxidative stress, and membrane permeability at each exposure scenario was normalized relative to the control. Based on the normalized data, the differences were considered statistically significant at \( p < 0.05 \) using Dunnett’s test [24].

3. Results and Discussion

Figures 3 and 4 show the biomass, cell size, cell granularity, esterase activity, oxidative stress, and membrane permeability of \( \textit{C. reinhardtii} \) grown on four exposure scenarios after 1, 4, 7, 16, 13, 16, 19, and 22 weeks. All but one endpoint showed no significant effects at the beginning of the experiment. The exception was the \( \textit{C. reinhardtii} \) grown on the fourth soil layer-treated group; it showed a decrease in cell size and cell granularity and an increase in membrane permeability. The third, second, and first soil layer-treated groups showed initial significant effects after seven, 10, and 10 weeks, respectively. Over time, all exposure scenarios consistently showed significant effects compared to the control.

The fourth soil layer-treated group showed a decrease in biomass after four and seven weeks. Cell size and cell granularity decreased after 1 week and increased after 13 weeks (cell size) and 16 and 19 weeks (cell granularity), respectively. The esterase activity decreased continuously after 10, 13, 16, and 19 weeks. Oxidative stress increased after 10 and 13 weeks. Membrane permeability also increased after 1, 13, 16, and 19 weeks. In the third soil layer-treated group, cell size and cell granularity increased after 10 weeks (cell size) and 16 and 19 weeks (cell granularity), respectively. Esterase activity decreased continuously after 10, 13, 16, and 19 weeks. Oxidative stress increased after 7, 13, 16, and 19 weeks. Membrane permeability also increased after 7, 10, 13, 16, and 19 weeks. In the second soil layer-treated group, cell granularity increased after 10 and 16 weeks. Esterase activity decreased after 13, 16, and 22 weeks. Oxidative stress increased after 13 and 16 weeks and membrane permeability increased after 10, 13, and 16 weeks. In the first soil layer-treated group, cell size and cell granularity increased after 10, 19, and 22 weeks (cell size) and 19 weeks (cell granularity), respectively. The esterase activity decreased after 10, 13, 19, and 22 weeks. Oxidative stress increased after 16 weeks, and membrane permeability increased after 13 and 16 weeks.

![Figure 3. Cont.](image-url)
Figure 3. Biomass and cell morphology of *Chlamydomonas reinhardtii* dependent on the time and contamination configuration in soil treated with silver nanoparticles (AgNPs). (a) Biomass, (b) cell size, and (c) cell granularity. Asterisks (*) represent results that are significantly different from the control ($p < 0.05$). N stands for “not applicable” due to low cell density.

The difference in the toxicity of the contamination configuration-dependent AgNP-treated soils can be attributed to the distance between the stressor (the soil layer) and the receptor (the *C. reinhardtii* grown on the disc that adhered to the topsoil). The initial effect on the *C. reinhardtii* grown on the fourth soil layer-treated group was faster than the other exposure scenarios, and third, second, and first soil layer-treated groups showed initial significant effects in that order. The toxic effects of AgNPs are known to be induced by the reactive surface area of nanoparticles (NPs) or silver ions dissolved from them [17,25,26]. In this study, the initial significant effect on the *C. reinhardtii* grown on the fourth soil layer-treated group may be related to how *C. reinhardtii* adhered to nanoparticles in the topsoil. Benoit et al. [27] reported strong sorption of Ag on soil ligands in the first few hours. The significant effect of AgNPs over time on the *C. reinhardtii* grown on the fourth, third, second, and first soil layer-treated groups may be related to the leaching of silver ions dissolved from the NPs. Previous studies also reported on the fate or toxicity of silver ions...
dissolved in soil from NPs over time [27–29]. Arenas et al. [28] reported that 25 and 75 nm PVP-AgNPs have low retention and high mobility in tropical soils. Diez-Ortiz et al. [29] reported that silver ions dissolved from NPs in 50 nm untreated AgNPs increased the toxicity of the latter for earthworms over time. Benoit et al. [27] reported that silver ions released from AgNPs at the end of their experiment were ten times higher than those at the beginning of the experiment.

![Graph](image-url)

**Figure 4.**
In terms of toxicity endpoints, a significant increase in cell size, cell granularity, oxidative stress, and membrane permeability and a decrease in esterase activity of *C. reinhardtii* were observed in all exposure scenarios at the end of the experiment. Some results related to cell size, membrane permeability, and esterase activity of this study were similar to those in our other recent study [17]. After six days of exposure to the AgNP-treated soil, *C. reinhardtii* showed an increase in cell size and membrane permeability and a decrease in esterase activity. According to prior literature, increase of cell size has been interpreted as production of exopolysaccharide enlarged from the algal cell wall as a protective barrier [17,30] or adhesion of nanomaterials to the algal cell wall [31]. Uptake of nanomaterials into algal cells has been interpreted as an increase of cell granularity [32,33]. The increase of oxidative stress has been regarded as a cellular antioxidant defense system to nanomaterials after disturbance of balance between oxidant/antioxidant processes [34]. Damage of membrane integrity and disturbance of electron and ion transport chains of the cell membrane after the attachment of nanomaterials has been regarded as increase of membrane permeability [35]. Decrease of esterase activity has been recognized as a reduction of metabolic activity in algal cells [36]. In AgNP-treated soils, NPs themselves or silver ions dissolved from the NPs may respond to exposed algal cell walls, thereby increasing cell size. Subsequently, membrane permeability may be increased due to attachment of NP or ions to algal cell walls and physical damage to the cell walls. After that, esterase activity may be reduced by toxicity of NP or ions internalized via permeable membrane, thereby increasing cell granularity. Finally, oxidative stress may operate against the toxicity of NP or ions in algal cells. In other words, the algae-soaked disc seeding assay can sense a change of subsoil contamination by periodic evaluation and multiple toxicity endpoints over time, although the algae-soaked disc is set on the surface soil.

However, further analysis on the toxic effects caused by AgNPs or dissolved Ag ions on *C. reinhardtii* is needed to clarity mechanism, as the mechanism of the experiment could not be determined in this study. In addition, an extended experiment using various soil algae and soil textures is needed to study, as the prior literature using an algae-soaked disc seeding assay shows morphological differences on *C. reinhardtii* and *Pseudokirchneriella subcapitata* exposed to nickel [20] and inapplicability on loamy sand soil due to high soil infiltration rate in a contaminated field [11].

![Figure 4. Physiological effects of *Chlamydomonas reinhardtii* dependent on the time and contamination configuration in soil treated with silver nanoparticles (AgNPs).](image)
4. Conclusions

This study applied the algae-soaked disc seeding assay to consider periodic evaluation of subsoil contamination under different contamination configurations of silver nanoparticle soils via multiple toxicity endpoints using the green alga C. reinhardtii. It enabled us to observe that the fourth soil layer-treated group showed initial significant effects faster than other exposure scenarios and the third, second, and first soil layer-treated groups showed initial significant effects in that order. A significant increase in cell size, cell granularity, oxidative stress, and membrane permeability and a decrease in esterase activity were observed in all exposure scenarios at the end of the experiment. These results may have been caused when the C. reinhardtii adhered to nanoparticles in the topsoil in the fourth soil layer-treated group, and silver ions dissolved from the NPs in the third, second, and first soil layer-treated groups leached into the soil. Therefore, the algae-soaked disc seeding assays can sense a change of subsoil contamination by periodic evaluation over time, because they were influenced by ionized pollutants that leached from the NPs distributed in subsoil. Overall, the results showed that the algae-soaked disc seeding assay is a useful method to evaluate the periodically rapid in situ toxicity of soil pollutants as a biomonitoring technique to measure subsoil contamination. However, further investigation is needed to study the use of various soil algae and soil textures and consider the toxicity mechanism and fate of soil pollutants in these cells and the soil matrix.

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References

1. Keesstra, S.D.; Geissen, V.; Mosse, K.; Piiranen, S.; Scudiero, E.; Leistra, M.; van Schaik, L. Soil as a filter for groundwater quality. Environ. Sustain. 2012, 4, 507–516. [CrossRef]
2. Bonaventura, R.; Zito, F.; Morroni, L.; Pellegrini, D.; Regoli, F.; Pinsino, A. Development and validation of new analytical methods using sea urchin embryo bioassay to evaluate dredged marine sediments. J. Environ. Manag. 2021, 281, 111862. [CrossRef] [PubMed]
3. Meier, J.R.; Chang, L.W.; Jacobs, S.; Toresella, J.; Meckes, M.C.; Smith, M.K. Use of plant and earthworm bioassays to evaluate remediation of soil from a site contaminated with polychlorinated biphenyls. Environ. Toxicol. Chem. 1997, 16, 928–938. [CrossRef]
4. Romero-Freire, A.; Fernández, I.G.; Torres, M.S.; Garzón, F.J.M.; Peinado, F.J.M. Long-term toxicity assessment of soils in a recovered area affected by a mining spill. Environ. Pollut. 2016, 208, 553–561. [CrossRef] [PubMed]
5. Wang, Q.-Y.; Zhou, D.-M.; Cang, L.; Sun, T.-R. Application of bioassays to evaluate a copper contaminated soil before and after a pilot-scale electrokinetic remediation. Environ. Pollut. 2009, 157, 410–416. [CrossRef]
6. Kwan, J.I.; Nam, S.-H.; Kim, S.W.; Bajagain, R.; Jeong, S.-W. Changes in soil properties after remediation influence the performance and survival of soil algae and earthworm. Ecotox. Environ. Saf. 2019, 174, 189–196. [CrossRef]
7. Cang, L.; Zhou, D.-M.; Wang, Q.-Y.; Wu, D.-Y. Effects of electrokinetic treatment of a heavy metal contaminated soil on soil enzyme activities. J. Hazard. Mater. 2009, 172, 1602–1607. [CrossRef]
8. Hammel, W.; Steubing, L.; Debus, R. Assessment of the ecotoxic potential of soil contaminants by using a soil-algae test. Ecotoxicol. Environ. Saf. 1998, 40, 173–176. [CrossRef]
19. Nam, S.-H.; Kwak, J.I.; An, Y.-J. Evidence for the inhibitory effects of silver nanoparticles on the activities of soil exoenzymes. *Chemosphere* 2012, *88*, 524–529. [CrossRef] [PubMed]

20. Nam, S.-H.; Kwak, J.I.; An, Y.-J. Quantification of silver nanoparticle toxicity to algae in soil via photosynthetic and flow cytometric analyses. *Sci. Rep.* 2018, *8*, 292. [CrossRef] [PubMed]

21. Shin, Y.-J.; Kwak, J.I.; An, Y.-J. Evidence for the inhibitory effects of silver nanoparticles on the activities of soil exoenzymes. *Chemosphere* 2012, *88*, 524–529. [CrossRef] [PubMed]

22. Baun, A.; Justesen, K.B.; Nyholm, N. Algal test with soil suspensions and elutriates: A comparative evaluation for PAH-contaminated soils. *Chemosphere* 2002, *46*, 251–258. [CrossRef]

23. Brussaard, C.P.D.; Marie, D.; Thyrhaug, R.; Bratbak, G. Flow cytometric analysis of phytoplankton viability following viral infection. *Aquat. Microb. Ecol.* 2001, *26*, 157–166. [CrossRef]

24. Dunnett, C.W. Multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* 1955, *50*, 1096–1121. [CrossRef]

25. Angel, B.M.; Batley, G.E.; Jarolimek, C.V.; Rogers, N.J. The impact of size on the fate and toxicity of nanoparticulate silver in aquatic systems. *Chemosphere* 2013, *93*, 359–365. [CrossRef]

26. Katsumi, A.; Gilliland, D.; Arostegui, I.; Cajaraville, M.P. Mechanisms of toxicity of Ag nanoparticles in comparison to bulk and ionic Ag on mussel hemocytes and gill cells. *PLoS ONE* 2015, *10*, e012903. [CrossRef] [PubMed]

27. Benoit, R.; Wilkinson, K.J.; Sauvé, S. Partitioning of silver and chemical speciation of free Ag in soils amended with nanoparticles. *Chem. Cent. J.* 2013, *7*, 75. [CrossRef] [PubMed]

28. Arenas, A.Y.; Pessôa, G.S.; Arruda, M.A.Z.; Fostier, A.H. Mobility of polivinylpyrrolidone coated silver nanoparticles in tropical soils. *Chemosphere* 2018, *194*, 543–552. [CrossRef]

29. Diez-Ortiz, M.; Lahive, E.; George, S.; Schure, A.T.; Van Gestel, C.A.M.; Jurkschat, K.; Svendsen, C.; Spurgeon, D.J. Short-term soil bioassays may not reveal the full toxicity potential for nanomaterials; Bioavailability and toxicity of silver ions (AgNO3) and silver nanoparticles to earthworm Eisenia fetida in long-term aged soils. *Environ. Pollut.* 2015, *205*, 191–198. [CrossRef]

30. Khona, D.K.; Shirolikar, S.M.; Gawde, K.K.; Hom, E.; Deodhar, M.A.; D’Souza, J.S. Characterization of salt stress-induced leucaconazole in a vineyard soil amended with spent mushroom substrate and its potential environmental impact. *Ecotox. Environ. Saf.* 2011, *74*, 1480–1488. [CrossRef]

31. Nam, S.-H.; Moon, J.; Kim, S.W.; Kim, H.; Jeong, S.-W.; An, Y.-J. Rapid in-situ assessment for predicting soil quality using an algae-soaked disc seeding assay. *Environ. Monit. Assess.* 2017, *189*, 637. [CrossRef] [PubMed]

32. Boxall, A.B.A.; Chaudhry, Q.; Jones, A.; Aitken, R.; Jefferson, B.; Watts, C. Current and Future Predicted Environmental Exposure to Engineered Nanoparticles; Central Science Laboratory, Department of the Environment and Rural Affairs: London, UK, 2007.

33. Arze, A.R.; Manier, N.; Chatel, A.; Mouneyrac, C. Characterization of the nano–bio interaction between metallic oxide nanomaterials and freshwater microalgae using flow cytometry. *Aquat. Microb. Ecol.* 2010, *54*, 191–198. [CrossRef]

34. Jagadeesh, E.; Khan, B.; Chandran, P.; Khan, S.S. Toxic potential of iron oxide, CdS Ag2S composite, CdS and Ag2S NPs on a fresh water alga Mougeotia sp. *Colloids Surf. B* 2015, 125, 284–290. [CrossRef]

35. He, X.; Xie, C.; Ma, Y.; Wang, L.; He, X.; Shi, W.; Liu, X.; Liu, Y.; Zhang, Z. Size-dependent toxicity of ThO2 nanoparticles to green algae Chlorella pyrenoidosa. *Aqua. Toxicol.* 2019, *209*, 113–120. [CrossRef]

36. Sousa, C.A.; Soares, H.M.V.M.; Soares, E.V. Chronic exposure of the freshwater alga Pseudokirchneriella subcapitata to five oxide nanoparticles: Hazard assessment and cytotoxicity mechanisms. *Aqua. Toxicol.* 2019, *214*, 105265. [CrossRef]