Cytotoxicity and genotoxicity of coated-gold nanoparticles on freshwater algae *Pseudokirchneriella subcapitata*

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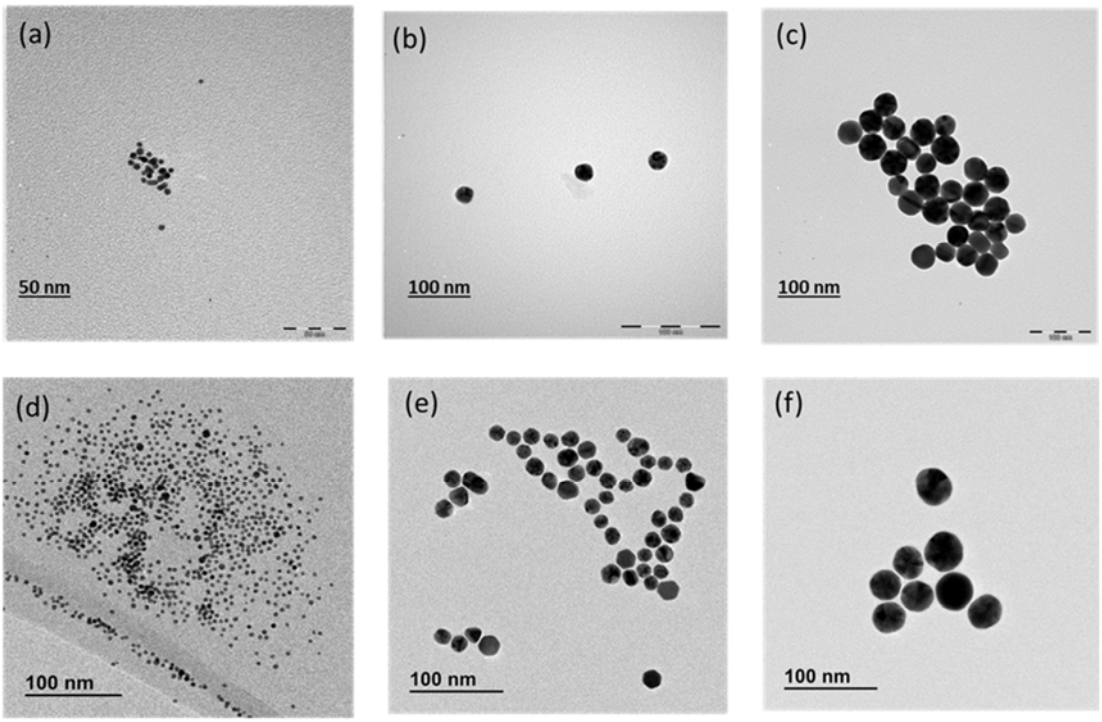

Figure S1: TEM images of nAu (a) 5 nm-Cit, (b) 20 nm-Cit, (c) 40 nm-Cit, (d) 5 nm-BPEI, (e) 20 nm-BPEI, and (f) 40 nm-BPEI (Mahaye, 2019).

SI-1 **Preparation of the algal test**

Inoculum was prepared by harvesting exponentially growing *P. subcapitata* from a 4 – 7 d old stock culture prepared in Section 2.2 under controlled conditions (temperature: 25 ± 1°C; light intensity: 6000 Lux; 12 h:12 h light: dark cycle, and shaken continuously at 100 rpm). The stock culture was transferred as 1 ml volume into 15 Eppendorf tubes and centrifuged at

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10 000 rpm for 10 min. The supernatant was decanted and the algal cells were re-suspended in 0.1 mL phosphate-buffered saline (PBS). The centrifugation and decanting steps were repeated twice. The volume of stock culture required, and cell density of algal inoculum required per experiment in test and control wells were calculated using the following expressions:

$$Volume\ (mL) = \frac{no.\ of\ flasks\ used\ X \frac{vol}{flask} X\ 200\ 000\ cells/mL}{Cell\ density\ (cells/mL)\ in\ the\ stock\ culture} \quad (1)$$

where vol/ flask is the volume of test solution per flask (USEPA 2002), and cells/mL is the cell density in the inoculum given by the following expression (Rodrigues et al., 2011):

$$Cells/mL = e^{\frac{ln\lambda_{684}+16.439}{1.0219}} \quad (2)$$

where λ_{684} is the optical density (OD) at 684 nm.

In the final step, algal cells were re-suspended and mixed well in 10% BG-11 algal media, and the cell density in the inoculum was measured before the experiment was initiated. For each test, 200 000 cells/mL sample was required. Tests were carried out in 2 mL volumes in 24-well microplates (1.8 mL test sample (or de-ionised water (DIW) for the control) plus 0.2 mL inoculum and algal medium) and incubated at the same conditions as the stock culture for 168 h. After the experiment was initiated, cell density (in the form of optical density) was measured at 684 nm every 24 h for 168 h using a microplate reader (FLUOstar Omega BMG LABOTECH). Briefly, the wavelength of 684 nm used here was adopted from Rodrigues et al., (2011) and has been successfully used on ENPs-exposed *P. subcapitata* studies (Grillo et al., 2015; Ozkaleli and Erdem 2018).

S1-2 Exposure medium preparation

Experiments were conducted using 10% BG-11 media following Direct Estimation of Ecological Effects Potential (DEEEP) toxicity testing protocols (Slabbert 2004). Chemicals for the BG-11 algal media; ($CuSO_4.5(H_2O)$, $ZnSO_4.7(H_2O)$, Co $(NO_3)_2.6H_2O$, $C_6H_8O_7$,

$NaHCO_3$, $NaNO_3$, $CaCl_2{\cdot}2(H_2O)$, $MgSO_4{\cdot}7(H_2O)$, K_2HPO_4, Fe (NH_3)-citrate, $Na_2EDTA{\cdot}2(H_2O)$, H_3BO_3, $MnCl_2{\cdot}4(H_2O)$, $Na_2MoO_4{\cdot}2(H_2O)$) were purchased from Sigma Aldrich (Johannesburg, South Africa) and were used without further purification. The BG-11 media chemicals were dissolved in 1 L DIW (15 MΩ.cm) in sterile autoclaved pre-cleaned glassware, the pH was adjusted to 7-7.5, and the media was autoclaved before used.

SI-3 Algal reference test with potassium dichromate

To assess the test sensitivity, the microplate algal growth inhibition assay (Slabbert, 2004) was performed using potassium dichromate ($K_2Cr_2O_7$) as a reference toxicant. Exponentially growing *P. subcapitata* were exposed to varied $K_2Cr_2O_7$ concentrations (0.32 -3.2 mg/L) over 72 h. Cell density readings were measured at OD_{684} every 24 h and the 50% effective concentration (EC_{50}) was determined. Herein, a concentration dependent growth inhibition of *P. subcapitata* due to $K_2Cr_2O_7$ compared to control was observed over 72 h as shown in Figure S2. EC_{50} value of 0.31 mg/L was determined, and considered to be in good agreement with 0.43 mg/L reported in the test kit.

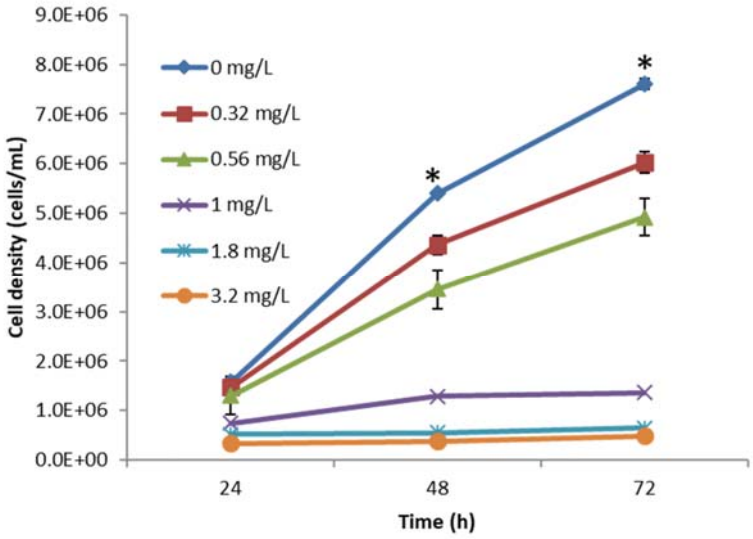

Figure S2: Algal growth of *P. subcapitata* at different concentrations of $K_2Cr_2O_7$. Bars denote standard deviation where n = 3, and * denotes significant differences (p < 0.05) between the control and treated samples.

SI-4 Cytotoxicity of nAu to *P. subcapitata*

SI-4.1 Effect of nAu on *P. subcapitata* growth

Exponentially growing *P. subcapitata* were exposed to five concentrations of nAu (62.5, 125, 250, 500 and 1 000 µg/L) for 168 h, in a 24-well microplate system, under defined conditions outlined in SI-1.

SI-4.2 Effect of nAu on *P. subcapitata* chlorophyll *a* content

Following the 72 h and 168 h exposure periods, chlorophyll *a* (Chl *a*) content was determined in accordance to the protocol by Harris (1989). Briefly, 1 mL of the control and exposed algal cells were centrifuged for 10 min at 13 000 rpm, and the pellet was washed using DIW. The algal cells pellet was suspended in 95% ethanol vortexed for 2 min, kept at 4 °C for 30 min and centrifuged at 13 000 rpm for 2 min. The supernatant was analysed for Chl *a* content using a UV-Vis spectrophotometer at wavelengths of 665 and 649 nm. The content of Chl *a* content was then calculated according to Equation 3:

$$\text{Chl a} = 13.70A_{665} - 5.76A_{649} \quad (3)$$

where A_{665} and A_{649} are the OD values (n =3) at wavelengths of 665 nm and 649 nm, respectively.

SI-5 Optimization of PCR conditions for RAPD assay

Random Amplified Polymorphic DNA (RAPD) PCR was performed with two 10-base pair RAPD primers; OPB1 (5'-GTTTCGCTCC -3') and OPB14 (5'-TCCGCTCTGG-3') purchased from Inqaba Biotechnical Industries (Pty) Ltd (South Africa). Amplification was performed in 25 µL reaction volumes consisting of 0.5 µL primers, 10 µL DIW, 12.5 µL GoTaq® G2 Hot Start Green Master Mix (GoTaq® G2 Hot Start Polymerase, dNTPs, $MgCl_2$ and reaction buffers: Promega, USA), and 2 µL genomic DNA using a Polymerase Chain Reaction (PCR) thermocycler (T100™ Thermal Cycler). For the negative control, genomic DNA was not added and DIW was used to make up the 25 µL volume. RAPD PCR conditions used by Wu et al. (2013) were followed. The PCR steps consisted of DNA denaturation for 5 min at 95 °C, followed by 30 cycles of 40 s at 95 °C, 40s at 56 °C, and 2 min at 72 °C, then final extension for 10 min at 72 °C. The amplified DNA was separated at 90 mV for 1 h and visualised using a UV transilluminator. Following these PCR steps, no bands were obtained

in 1% and 1.5% ethidium bromide-stained agarose gel dissolved in 1 X TAE buffer (Tris-acetate-EDTA buffer, pH 7.5).

A gradient PCR was then used to find optimum annealing temperatures for each primer. A temperature range between 35 and 50° C was selected based on primer melting temperatures as provided by the supplier (39.5 °C for OPB1 and 43.6 °C for OPB14). The annealing temperatures tested for each primer were in the range of 35- 50 °C. The RAPD-PCR protocol consisted of the following steps: a 35-cycle warming step at 95 °C for 5 min, DNA denaturation at 95°C for 1 min, annealing (30 –50 °C) for 1 min, extension 74 °C for 1 min, and final extension at 74 °C for 10 min. The amplified product was gel electrophoresed at 80 mV for 2 h and visualised using a UV transilluminator. No DNA bands were observed at any of the tested temperatures. Following the same procedure, the study was repeated with bovine serum albumin (BSA) to enhance binding between the DNA and primer at an annealing temperature range of 35–50 °C (50.0, 48.7, 46.8, 44.3, 40.8, 37.8, 35.9, 35.5 and 35.0 °C). A comparison of results generated with and without BSA, showed the former had clear bands, and hence BSA was used in this study as DNA-primer binding enhancer. The annealing temperatures (46.8 °C for OPB1 and 44.3 °C for OPB14) with more visible bands for each primer were selected for the experiments.

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