PHENOTYPIC RESPONSES OF GREEN ALGA CHLAMYDOMONAS REINHARDTII TO NANOSCALE ZERO VALENT IRON

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Abstract

The toxicity of two iron-based nanoparticles: nFe_3O_4, and nZVI were assessed on freshwater microalgae, *Chlamydomonas reinhardtii* CC-5325. Microalgae response (total chlorophyll/carotenoids content, photosystem II efficiency, cell shape and total viable cell numbers) to the nanoparticles exposure (100 mg/L) was monitored up to 120 hours. Based on phytochrome and photosystem II analysis performed, almost no significant impact was found. However, microscopic analysis and the total viable cell numbers revealed a certain degree of inhibition effect showing altered cell shape, and higher number of dead cells after the exposure to both nanomaterials. The dead cell numbers increased within one hour after the exposure to nFe_3O_4, while nZVI caused rather slow inhibition effect and persisted until 48 h with the highest dead cell number. In a series of experiments performed, the results may justify that exposure of these two NPs initially slightly inhibited *C. reinhardtii*, however the culture was able to recover towards the end of the study, because of new cell generation and nZVI oxidation.

Keywords: Metal oxide nanoparticles, nZVI, algae, physiological effect

1. INTRODUCTION

Nanoscale zero-valent iron (nZVI) is a strong reducing agent which has been used for more than a decade mainly for environmental remediation to clean up polluted aquifers [1–3] and was also suggested for elimination of harmful cyanobacterial blooms (REF) [4]. Potential application of nanoscale magnetite (nFe_3O_4) could be used as drug delivery system, contrast agent for magnetic resonance imaging, or hyperthermia agent in cancer treatment [5]. To date, the toxicity of iron nanoparticles have been evaluated on numerous cell lines and microorganisms [6–8]. Although different parameters were measured, they were mostly interested in describing the effect of different nanoparticles concentrations rather than in establishing mechanistics links between the measured functions. Thus, in this study, effect of two differernt iron nanoparticles (i) reactive nZVI and (ii) non-reactive nFe_3O_4 was assessed on *Chlamydomonas reinhardtii* which is a model organisms used in many toxicological studies [9]. In particular, several phenotypic effects of *C. reinhardtii* were studied upon these NPs exposure, such as photosystem II efficiency, cell shape and total viable cell count.

2. MATERIALS AND METHODS

2.1. Preparation of nZVI and nFe_3O_4

The supplied NANOFER STAR powder (nZVI) (CAS no: 7439-89-6, size 10-150 nm) and nanoscale magnetite (nFe_3O_4) (CAS No: 1317-61-9, size 50-100 nm) were obtained from NANOIRON s.r.o, Czech Republic, and Sigma Aldrich, Merck, Germany, respectively. Activation of 20% NPs suspension were done according to producer guidelines for 48 h [10].

2.2. Culture condition of *C. reinhardtii* and experimental setup

Chlamydomonas reinhardtii CC-5325, was obtained from the *Chlamydomonas* Resource Center University of Minnesota (USA). The culture was cultivated under a 130 µmol photons·m^{-2} s^{-1} of constant white light illumination and gently bubbled by 1% CO_2 mixed with air at room temperature (24 °C). The cultivations were conducted in a 500 mL flask containing Sueoka's high salt medium (HSM) [11]. A preculture of algae in HSM was started 48 h before addition of nanoparticles (–NPs). At time point t = 48 h in (–NPs), the pre-cultured algae were then harvested from batch culture during mid-log exponential growth (at an initial cell density of 6.026 × 10^6 cells/mL), then added to a flask containing NPs, which is labelled as (+NPs). Three different experimental conditions were set up, namely the: i) unexposed *C. reinhardtii* (control), ii) *C. reinhardtii* with 100 mg/L of nFe_3O_4 and iii) *C. reinhardtii* with 100 mg/L of activated nZVI.

Three replicates for each condition were conducted in 550 mL of HSM, starting with 45 mL of pre-grown algae culture. Total nanoparticle-algae exposure hours were up to 120 h from t = 0 h in (+NPs), under photoautotrophic conditions as mentioned above. Cultures (15 mL) were sampled every 24 h before +NPs exposure (up to 48 h, from –NPs pre-grown culture) and after the start of +NPs exposure at 0, 1, 4 h and thereafter every 24 h up to 120 h for microscopy, viable cell count, chlorophyll and carotenoids and photosystem II analysis. All the measurements were done in three technical replicates per biological sample for each time point and averaged prior to data analysis.

2.3. Phenotypic analysis

2.3.1. Light microscopy, and viable cell count

Algae cell distribution was imaged under optical microscopy (Motic BA310, Motic, China) to assess the substantial physical interaction, NPs aggregation and calculation of cell number. The cell number was determined by manual cell counting using a Neubauer haemocytometer (Superior Marienfeld, Germany).

2.3.2. Chlorophyll quantification and fluorescence analysis

The extraction of photosynthetic pigments (total chlorophyll and carotenoids) were carried out with 90% methanol as described by Lichtenthaler [12] and calculated according to Wellburn [13].

2.3.3. Photosystem II efficiency

Photosystem II efficeincy was measured in a 48-well flat bottom plate using a closed FluorCam FC 800-C Video Imager (Photon Systems Instruments, Czech Republic). Before the measurement, samples were dark-adapted for at least 15 min. The parameters F_v/F_m, non-photochemical quenching (NPQ), PSII photochemistry were calculated as described by Murchie [14].

2.3.4. Statistical analysis

Statistical analysis was performed using Prism (GraphPad, USA). The mean differences of the treatment groups were compared against a *C. reinhardtii* control respectively by two-way ANOVA and Dunnett's multiple comparison test. The differences were considered statistically significant at *P< 0.05, **P < 0.001, ***P < 0.0001.

3. RESULTS AND DISCUSSION

3.1. Morphology changes of algae upon NPs exposure

The light microcopy images (**Figure 1**) show different cell stages and interaction between algae cells and NPs. The cells in the control had intact plasma membrane closely attaching to the cell wall with two flagellar tails (**Figure 1, A1-A3**), and new cells started to divide after 24 h (**Figure 1, A4**). A strong NPs attachment on algae was observed from time zero (**Figure 1, B1, C1**), and cells were slightly deformed or lysed which was observed from 48 h (**Figure 1, B5, C5**) or cell lost their chlorophyll pigments leaving empty cells/cell debris behind (**Figure 1, C2**). Such a cell alteration also appeared in a previous study [7]. At immediate exposure, there was small degree of chlorophyll lost in nFe_3O_4 indicated by the yellow arrows which was not seen in nZVI exposure.

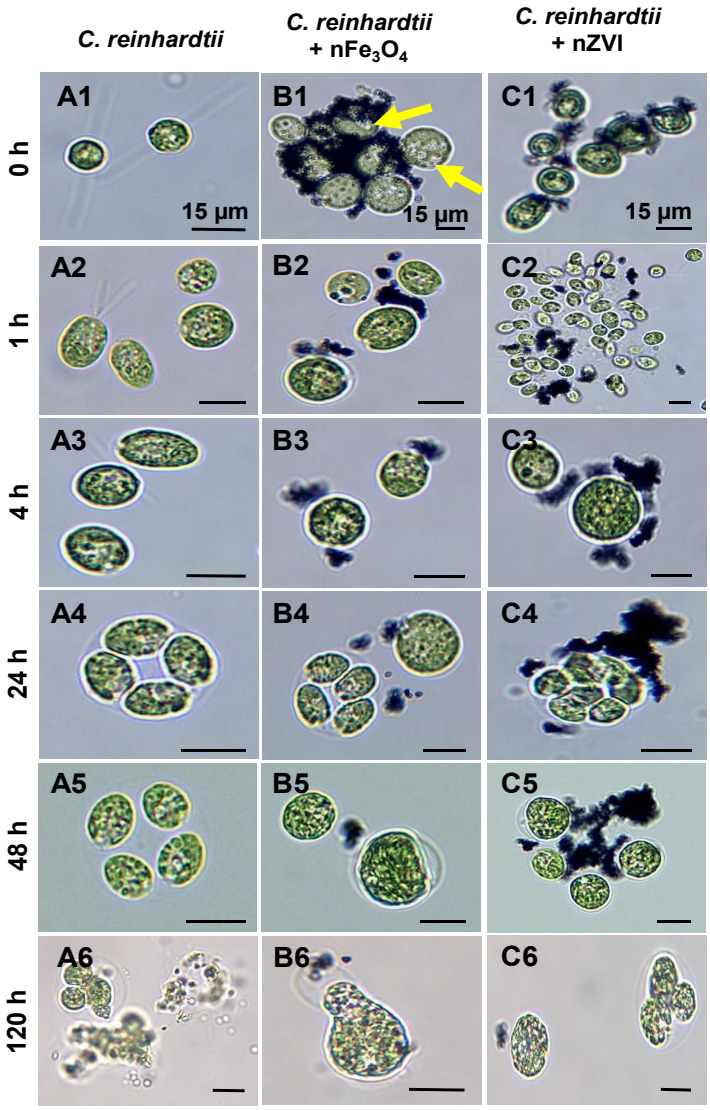

Figure 1 Microscopic imaging of *C. reinhardtii* exposed to nFe_3O_4 and nZVI up to 120 h. (A) Untreated *C. reinhardtii*; (B) *C. reinhardtii* exposed to 100 mg/L nFe_3O_4 (C) *C. reinhardtii* exposed to 100 mg/L nZVI. Scale bar = 15 µm.

3.2. Phenotypic responses of algae upon NPs exposure

The overall phenotypic responses of *C. reinhardtii* was not statistically signicant until 24 h (**Figures 2A-2D**), except NPQ (**Figure 2E**). In the nZVI (indicated by red line), the NPQ value was the lowest comparing three

exposure conditions and was significantly affected within 1 h, and these NPQ values continued to decrease until 48 h, and thereafter increased sharply by 120 h, suggesting that cells may be at stress from the initial NPs exposure while were able to recover from the NPs stress after several days. Phenotypic results summarized in (**Figure 2**) display almost no detrimental effect of NPs to *C. reinhardtii*, as their values are similar to control condition (green line). Furthermore, the smaller fluroescence intensity of photosystem II observed in both NPs treatments could be partly due to the shading effect of NPs (**Figure 2F**), whereas *C. reinhardtii* almost regained its photosystem II after 120 h. Exposure to NPs would also caused oxidative stress or DNA damage in cells, due to the oxidation of nZVI and generation of hydroxyl radical [15]. Standard toxicity endpoints are depending mostly on the phenotypic responses of microorganisms such as growth inhibition, or oxidative stress formation. To comprehend the underlying stress beneath the cell membrane on how the cell would defend oxidative insults, more toxicity endpoints are needed for evaluation, such as transcriptomic analysis to better reveal hidden pattern of cell defense [16,17].

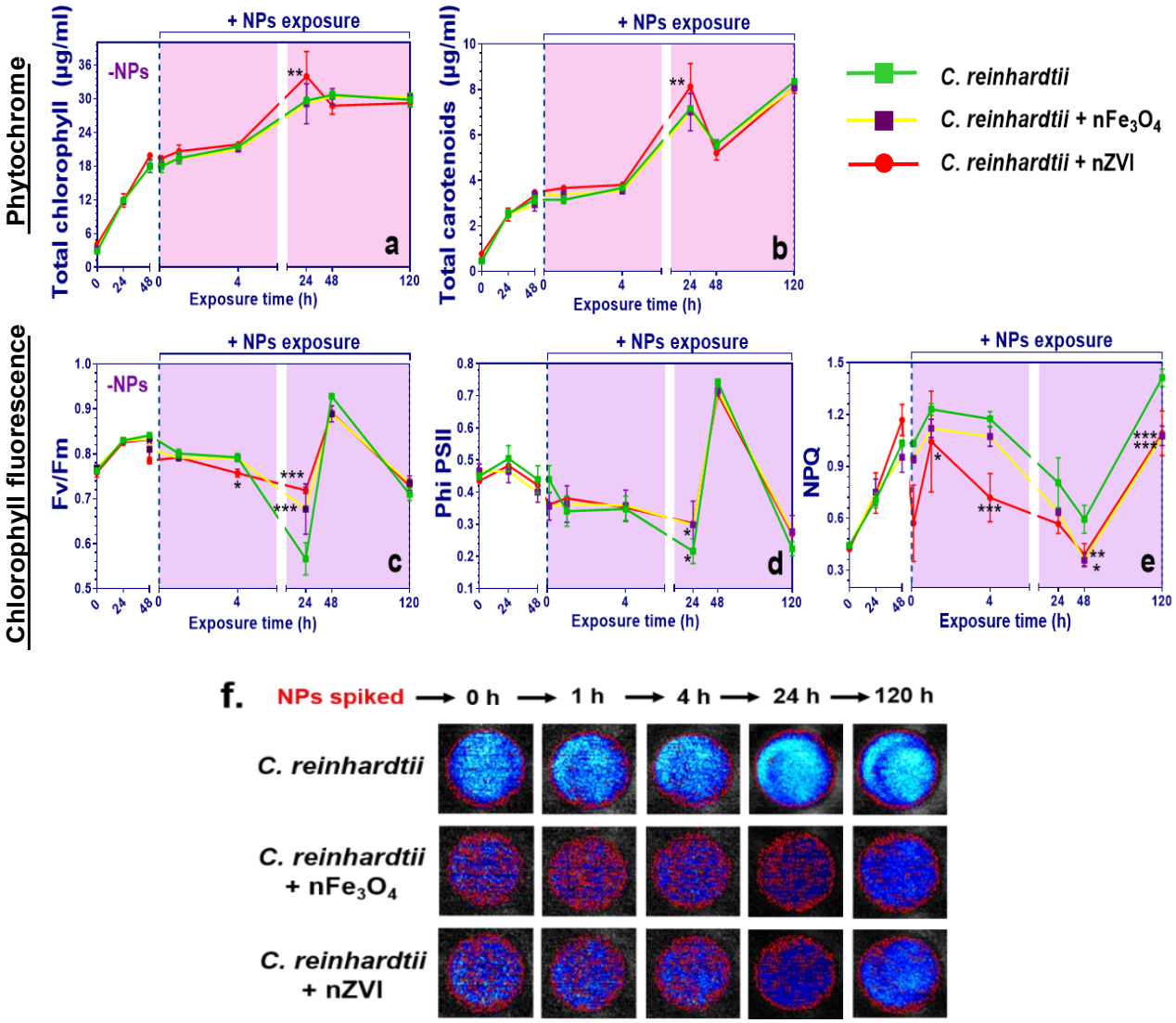

Figure 2 Phenotypic responses of *C. reinhardtii* exposed with nZVI and nFe_3O_4 in HSM. (A,B) Phytochrome content (Total chlorophyll and carotenoid); (C,D,E) Photosystem II efficiency (F_v/F_m, non-photochemical quenching parameters, PSII photochemistry), (mean ± SD; n = 3); (F) Light intensity of chlorophyll fluorescence imaged under FluorCam FC 800-C Video Imager. *= level of significance changes were compared to untreated exposure medium containing only *C. reinhardtii* (*C. reinhardtii,* solid green line), (*P< 0.05, **P < 0.001, ***P < 0.0001). Note: different scales for the y-axis.

3.3. Viable cell numbers of algae upon NPs exposure

The effect of both NPs on *C. reinhardtii* cells was assessed by counting the viable cell (intact cell) and dead cell (cell lost its pigment/plasmolyzed cells) and the results are shown in (**Figure 3**). Both NPs inhibited the algae cell as observed by the reduction of cell viability, however, in a different pace of action. The cell viability in the control was stable with more than 80% viable cells during the whole experiment (**Figure 3A**). nFe_3O_4 caused a decrease in viability by 10% comparing to the control - approximately 69% viable cells were recorded after one hour and this proportion persisted until the end of the study (**Figure 3B**). Effect of nZVI was rather slow and the highest proportion of dead cells occured after 48 h – 30% decrease in viability comparing to control (**Figure 3C**).

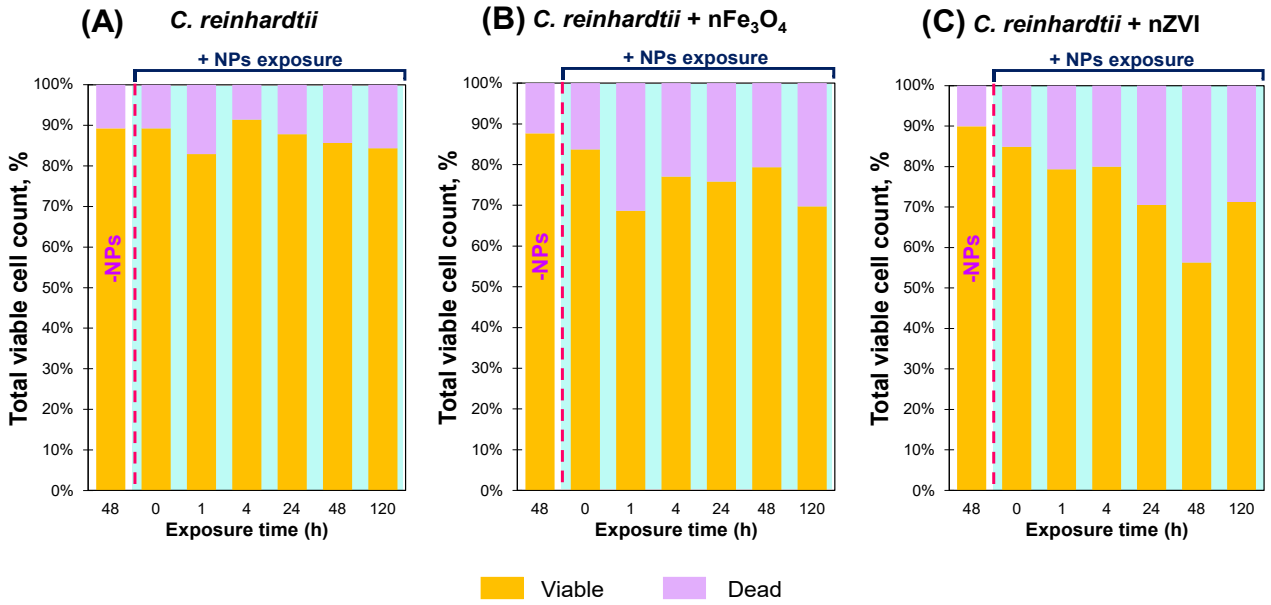

Figure 3 Total viable intact cell and dead cell, determined by manual counting using haemocytometer under light microscope. Values were expressed in percentage, (mean; n = 3).

4. CONCLUSION

Iron NPs caused minor negative effect to *C. reinhardtii* during the initial hours of exposure, while the culture was able to recover towards to the end of the study, probably due to nZVI oxidation, harmless iron oxides and new cell generations. This study amends the current knowledge about the potential risks of nZVI application in the environment.

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