Comparison of the Effects and Distribution of Zinc Oxide Nanoparticles and Zinc Ions in Activated Sludge Reactors

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Abstract

Zinc Oxide nanoparticles (ZnO NPs) are increasingly applied in the industry which results inevitably in their release of these materials into the hydrosphere. In this study, simulated waste activated sludge experiments were conducted to investigate the effects of Zinc Oxide NPs and compare it with its ionic counterpart (as $ZnSO_4$). It was found that even 1 mg/L ZnO NPs could have a small impact on COD and ammonia removal. Under 1, 10 and 50 mg/L ZnO NPs exposure, the Chemical Oxygen Demand (COD) removal efficiencies decreased from 79.8% to 78.9%, 72.7% and 65.7%, respectively. The corresponding ammonium (NH_4-N) concentration in the effluent significantly ($p < 0.05$) increased from 11.9 mg/L (control) to 15.3, 20.9 and 28.5 mg/L, respectively. Under equal Zn concentration, zinc ions were more toxic towards microorganisms compared to ZnO NPs. Under 50 mg/L exposure, the effluent Zn level was 5.69 mg/L, implying that ZnO NPs have a strong affinity for activated sludge. The adsorption capacity of ZnO NPs onto activated sludge were found to be 2.3, 6.3, and 13.9 mg/g SS at influent ZnO NP concentrations of 1.0, 10 and 50 mg/L respectively, which were 1.74, 2.13 and 2.05 fold more than under Zn ions exposure.

Keywords: ZnO nanoparticles; zinc ions; waste activated sludge; biosorption;

Introduction

Nanotechnology has become very popular over the last few decades due to significant advances with applications in medicine and semiconductor, chemical and electronics industries. [1, 2] Zinc oxide (ZnO) nanoparticles (NPs) is one of the most important engineered metal-oxide NPs in electronic sensors, solar cells, coatings, pigments and optics due to its semiconductors properties

such as near UV emission and transparent conductivity. [3, 4] They are also applied for the oxidation of environmental pollutants and personal care products and as disinfectants in medicine due to their unique photolytic properties. [5]

It is reasonable to believe that an increase in their production and application in the modern industries will inevitably result in their release into the environment and in particular in our waterways. [2, 6] Wastewater treatment plants are considered the last barriers prior to the environmental release of engineered NPs. [7] An environmentally relevant concentration of ZnO NP in wastewater would be around 24-300 µg/L according to Sun et al. [8], but the concentration is likely to be in the mg/L level in the next few years.[9]

Furthermore, ZnO NPs are one of the most toxic NPs produced. [10, 11] Farre et al. [12] reported the half maximal effective concentration (EC50) to be in the range of tens of µg/L to several mg/L. Their toxicity on bacteria and crustaceans was demonstrated with LC50 ranging from 0.1 to 10 mg/L for ZnO NPs as well as $ZnSO_4$.[13, 14] The exact toxicity of NPs and ionic counterparts on waste activated sludge is still not clear.

In this regard, the potential impact of ZnO NPs on the microbial community in wastewater treatment processes have drawn increasing concern because biological treatment of wastewater relies on bacteria to decompose organic matter and nitrogen compounds. In addition, the fate, transport, and toxicity of NPs in wastewater treatment processes may differ largely from those of their ionic counterparts, due to the differences in size and surface charge, potential for biosorption or aggregation. [7] However, to date, knowledge on the fate and transformation of ZnO NPs in wastewater treatment processes is still scarce. [15, 16] Interactions with natural organic matter in real wastewater may result in different behaviour of Zn NPs. For instance, Zn ions can generate complex with humic acids due to their carboxylic and phenolic groups or precipitate as insoluble zinc hydroxide. Moreover, there is evident discord in the published literature regarding the fate and behaviour of ZnO NPs, [17] as well as how this influences their toxicity. [18]

The objectives of this study were (a) to compare the short term effects and fate of ZnO NPs and Zn^{2+} ions in a laboratory scale waste activated sludge process using sequencing batch reactor (SBR) fed with real wastewater; (b) to investigate the effects of 1, 10 and 50 mg/L ZnO NPs on COD and nitrogen removals; (c) to determine the accumulation of Zn ions in the effluent and onto activated sludge over short term experiments; (d) to determine the morphology of activated sludge using Scanning electron microscopy (SEM); (e) to assess the impacts of the presence of ZnO NPs and Zn^{2+} ions on bacterial integrity using the Live/Dead *Bac*light bacterial viability technique which was not used previously in particular under short term experiments (5 hours) at concentrations as high at 50 mg/L.

Materials and methods

Activated sludge samples

Primary wastewater was collected from Ulu Pandan Water Reclamation Plant (WRP), Singapore. The total treatment capacity of Ulu Pandan WRP is 361,000 m^3 per day. The treatment process includes typical preliminary, primary and secondary treatment processes. The wastewater was collected from the effluent of the primary sedimentation tank. As Ulu Pandan WPR treats combined industrial and domestic wastewater, the contaminant concentrations are expected to be higher than those in common domestic WWTPs. Real wastewater was stored at 4°C until it was fed to the SBRs.

Set-up of Sequencing Batch Reactors (SBR)

SBRs were designed to simulate a full-scale operation of aeration and secondary clarification as described by Hou et al. [19] Briefly, SBRs were set up in 500 mL glass beakers as reactors, which were continuously operated for 15 days at 12 hours hydraulic retention time, allowing acclimatization to reach a stable performance. The steady state was established by monitoring the chemical oxygen demand (COD), ammonium and phosphate removal. The SBR cycle consisted in aeration for 10 hours, followed by settling for 2 hours. The SBRs were seeded with nitrifying sludge from Ulu Pandan WRP and adjusted to a mixed liquor suspended solid (MLSS) concentration of 3 g/L, using the effluent from the primary clarifier at the same plant. In each cycle, supernatants following settling were replaced with the effluent from the primary clarifier to start the next cycle.

After 15 days of stabilisation period, three SBRs were spiked with ZnO NPs at the concentrations of 1.0, 10, and 50 mg ZnO/L, respectively and three SBRs were spiked with corresponding ionic salt (in the form of $ZnSO_4 \cdot 7H_2O$) at concentration of 3.54, 35.4, and 177 mg $ZnSO_4 \cdot 7H_2O$/L such that both sets of SBR contained exactly 0.8, 8.0 and 40.0 mg Zn/L, respectively. One SBR was employed as control with no Zinc addition. Each condition was operated for one month and steady-state data were collected over three cycles to determine average and standard deviation.

ZnO NPs characterization

The ZnO NPs were purchased from Sigma-Aldrich (Singapore) with an average particle size of 40±5 nm. ZnO NPs stock solutions (100 mg/L) were prepared by adding dry particles into Milli-Q (pH=6.8±0.2), ultrasonicating the suspensions (30°C, 100 W, 40 kHz) for 30 min and shaking for 2 h to increase their dispersion. The particle-size distribution and *zeta* potential of ZnO NPs in the suspensions during 24-h incubation were measured using a Malvern Zetasizer Nano-ZS

(Malvern Instruments Ltd., UK). The morphology of the ZnO NPs was examined using transmission electron microscopy (TEM) (JEOL JEM-3010, Japan). To avoid agglomeration or aggregation, water bath ultrasonic treatment was carried out to increase their dispersion before using the ZnO NPs suspension.

Analytical methods

Sampling commenced after 15 days of operation of reactor, in order to ensure stable operation. Aliquots of completely mixed liquor suspensions were collected every 0.5 h over a period of 5 h. Collected samples were first centrifuged for 20 min at 10,000 rpm (Eppendorf 5810R). The supernatant was collected and the concentrations of COD, MLSS, ammonium (NH_4-N) and phosphate (PO_4^{3-}) were determined according to Standard Methods.[20] All chemical tests were done in duplicate.

Analysis of the released Zn^{2+} concentration in the supernatant was conducted after centrifugation (10,000 rpm for 20 min). 0.5 mL of the supernatant was added to 4.5 mL of Milli-Q water containing 2% ultra-high purity HNO_3. [21] The resulting Zn^{2+} concentrations in the supernatant were measured by MP-AES (4100, Agilent Technologies) in triplicate.

In addition to the liquid samples, the Zn level in the activated sludge was also analyzed after acid digestion. The mixed liquor was first centrifuged at 10,000 rpm for 20 min (Eppendorf 5810R) and the supernatant was removed. A 0.5 g sample of solid sludge was totally digested with 3 mL nitric acid (69%, Sigma-Aldrich) followed by 1 mL hydrochloric acid (37%, Sigma-Aldrich) at 105ºC for 2h, followed by filtration through a 0.45 µm filter membrane (Whatman, USA). The resulting solution was diluted to a final volume of 10 mL using Milli-Q water. The Zn^{2+} level in the resulting solution was measured by MP-AES.

150 ***Bacterial viability assay***

151

152 In order to shed light on the impact of ZnO NPs and zinc ions on bacteria integrity, *Bac*light
153 LIVE/DEAD bacterial viability kit was used (Molecular Probes, USA) as previously
154 described.[22]

155

156 ***Scanning electron microscopy (SEM) and transmission electron microscope (TEM) imaging***

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158 Samples were investigated using TEM and SEM. In the first case TEM, grids were prepared by
159 placing a drop of suspension (mixed liquor or supernatant) on a holey carbon grid and drawing
160 the suspension through the TEM grid using a paper tissue. The TEM grids were washed
161 afterwards in a drop of distilled water to remove the dissolved compounds. [23] The TEM was
162 operated at 200 kV to detect and characterize aggregation state of NPs in the solution.
163 To prepare SEM image, mixed liquor was first washed 3 times with 0.1 M phosphate buffer
164 solution (PBS) (pH 7.7) and fixed in 0.1 M phosphate buffer (7.4) containing 2.5%
165 glutaraldehyde at 4 °C for 4 h. The dried samples were coated with platinum before SEM
166 analysis according to Zheng et al.[21] The elemental analysis of the particles was carried out using
167 an energy-dispersive X-ray spectroscope (EDS).

168

169 ***Statistical analysis***

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171 The average ± standard deviation (SD) were reported for each concentration. In order to
172 determine the statistical significance between treatments the critical values through ANOVA
173 one-way analysis of variance were compared (SPSS Statistics V17.0). Results were deemed
174 different at $p < 0.05$.

Results and discussions

Characterization of engineered ZnO NPs

Figure 1 shows ZnO NPs in deionized water imaged by TEM with different scales (i.e., 0.5 µm and 500 nm). In the present study, due to their small size and huge surface area, ZnO NPs tend to aggregate or agglomerate in aqueous phase. Although the ZnO NPs used in this study have a diameter in the range of nanometers, some aggregates of different sizes formed in the particle suspension, even after sonication. The size distribution of ZnO NPs is presented in Supplementary Figure S1. The size ranged from 15 nm to 47 nm with a mean size of 33± 8 nm (n=107), which confirmed the nano size range. The *zeta* potential was found to be -11.7 mV at pH= 6.8 and -6.3 mV at pH=6.4 at the beginning and end of the experiment, respectively.

Removal of ZnO NPs and zinc ions in the activated sludge process

The Zn level in the biomass-free effluent is shown in Figure 2. After 5 h exposure (300 min), the concentrations of soluble Zn^{2+} in the effluent were 0.11, 1.19 and 5.69 mg/L at the initial ZnO NP concentration of 1.0, 10 and 50 mg/L, respectively. The higher concentrations of released Zn^{2+} observed at the initial ZnO NP concentration of 50 mg/L might have been attributed to the increased sludge surface charge and the decreased hydrophobicity resulting in more zinc ions being released from ZnO NPs. [24] Interestingly, the released the Zn^{2+} levels in Zn^{2+} ionic treatment (Figure 2B) (0.19, 2.15 and 9.41 mg/L, respectively) were significantly higher than

those in the NP treatment indicating that dissolution of Zn^{2+} was prevalent with $ZnSO_4$. Less Zn^{2+} was released from NP because humic acids are known to stabilize ZnO NP and retard dissolution rates.[25] By comparison, in a recent study on the fate and behaviour of ZnO NPs in a simulated WWTP, Musee et al. [17] reported an effluent Zn concentration of 1.39 mg/L after 240 hours of exposure. In the present study at 5 hours exposure, 86.3%, 85.1% and 85.8% of zinc from ZnO NPs were retained in the sludge at initial ZnO concentrations of 1.0, 10 and 50 mg/L respectively, showing that a large fraction of the ZnO NPs was removed from the wastewater due to adsorption onto waste activated sludge. In contrast, Zn^{2+} treatment exhibited lower removal efficiencies of 76.3%, 73.1% and 71.2%, compared to ZnO NP treatment.

Effect of ZnO NPs and Zn^{2+} ions on COD removal

Prior to addition of ZnO NPs, the COD concentration in the effluent was around 66 mg/L (Figure 3) which corresponds to removal efficiency of 79.8%. However, the presence of ZnO NPs even at 1 mg/L influenced the COD removal efficiencies, which slightly decreased to 78.9% ($p < 0.05$). The exposure to 10 and 50 mg/L ZnO NPs further decreased COD removal efficiencies to 72.7% and 65.7%, respectively. This is in disagreement with Chauque et al. [25] who reported no effect on COD removal at 20 mg/L ZnO NP. Our findings contradict previous studies of the effects of ZnO NPs on COD removal efficiencies. [16, 17] Tan et al. [26] investigated long-term (240 days) effects of ZnO NPs on the system performance of a membrane bioreactor (MBR) and reported that both short- and long-term exposure to 1.0 $mg.L^{-1}$ of ZnO NPs did not significantly affect COD removal, despite the fact that ZnO NPs may exhibit toxic effects on microorganisms. Likewise, Puay et al. [16] evaluated the effects of ZnO NPs on system performance and bacterial

224 community dynamics of biological wastewater treatment in a lab-scale SBR (over 62 days), and
225 indicated that the removal of COD was not affected significantly by 1 mg/L ZnO NPs.
226
227 However, in the present study, negative impacts on COD removal efficiencies were indeed
228 observed at ZnO NPs concentrations as low as 1 mg/L. This findings suggest that industries
229 releasing high amounts of Zinc nanoparticles should capture NPs before their release or dilute
230 their effluent accordingly to avoid negative impacts on the waste activated sludge process. The
231 lower COD removal efficiencies in the presence of ZnO NPs at higher concentrations are mainly
232 attributed to the Zn^{2+} released from the ZnO NPs, and the high toxicity of the increasingly
233 abundant Zn^{2+} ions from ZnO NPs at higher concentrations further reduced the ability of
234 microorganisms to oxidise organic matter. [16, 26] Furthermore, efficient aggregation and proper
235 settling of flocs is of significant importance for the generation of good-quality effluent in the
236 activated sludge process. [27]
237 At concentrations of 10 and 50 mg/L, $ZnSO_4$ exhibited lower COD removal of 68.2% and 42.7%,
238 compared to those of 72.7% and 65.6% in the presence of ZnO NPs. This finding suggests that
239 compared to ZnO NPs, Zn^{2+} ions exhibited acute toxicity towards microbes at high
240 concentrations, resulting in more severe inhibition of microorganisms. From Figures 2 and 3, it is
241 clear that ZnO NPs is less toxic than $ZnSO_4$ due to the fact that Zn ions from $ZnSO_4$ dissolve
242 more readily in water. Our findings are not in line with Heinlaan et al. [28] who reported that nano
243 ZnO and $ZnSO_4$ exhibit similar toxicities to *Vibrio fischeri* (with LC_{50} of 1.1 versus 1.9 mg/L),
244 *Daphnia magna* (6.1 versus 3.2 mg/L) and *Thamnocephalus platyurus* (0.98 versus 0.18 mg/L).
245 Liu et al. [18] also suggested that the IC50 values of soluble Zn on activated sludge endogenous
246 respiration, BOD biodegradation, ammonia oxidation, and nitrite oxidation were 2.2, 1.3, 0.8,
247 and 7.3 mg-Zn/L, respectively. In this study, after the addition of 50 mg/L ZnO NPs (equivalent
248 to 40 mg/L Zn^{2+}), the measured Zn^{2+} concentration in the effluent progressively increased to

only 5.7 mg/L after 5 hours, indicating a low dissolution potential of ZnO NPs in the system, a finding consistent with a previous study.[21] However, it is likely that 5.7 mg/L was causing some inhibition regardless of Zn ions origin which contradicts Hou et al. [29] who did not report reduced COD removal at 5 mg/L. This can be explained by the fact that short term experiment using non-acclimatized sludge were performed in this study. When $ZnSO_4$ was used, the Zn^{2+} concentration quickly increased to 6.5 mg/L after only 30 minutes and gradually increased to 9.4 mg/L after 300 minutes, which resulted in a greater toxicity.

Effect of ZnO NPs and Zn^{2+} ions on NH_4^+-N removal

The effects of ZnO NPs and Zn^{2+} ions on NH_4^+-N removal are shown in Figure 4. Prior to the ZnO NP exposure, the NH_4^+-N removal efficiency was 70.3%, but decreased to 63.8% in the presence of ZnO NP at 1 mg/L. Under 10 and 50 mg/L ZnO NPs exposure, the effluent NH_4^+-N significantly ($p < 0.05$) increased from 11.9 mg/L (control) to 20.9 and 28.5 mg/L, respectively. This finding implies that the decrease in NH_4^+-N removal correlate with the inhibition of nitrifying bacteria in the biomass even at low dose of ZnO NPs which was not reported previously using real wastewater. Zheng et al. [21] evaluated the effects of ZnO NPs on wastewater biological nitrogen removal by carrying out a short-term study (4.5 h) in a SBR, and reported that the presence of 10 and 50 mg/L ZnO NPs decreased total nitrogen removal from 81.5% to 75.6% and 70.8%, respectively. Likewise, Tan et al. [26] indicated that a significant decrease ($p < 0.05$) in NH_4^+-N removal was observed after ZnO NP exposure at concentrations of 1.0 mg/L and 10.0 mg/L ZnO NPs (from 89.9% to 87.2% and 85.2%, respectively). Hou et al. [29] indicated that even low ZnO NP concentrations of 5 mg/L exhibited a significantly negative effect on NH_4^+-N removal in a simulated SBR process with an 11-d operation period, and observed an 23.7% inhibition in nitrification during exposure to 5.0 mg/L ZnO NP. Additionally,

in the present study, effluent ammonia concentrations (18.7 mg/L, 29.3 mg/L and 35.2 mg/L, respectively) in the presence of $ZnSO_4$ were higher than those in the presence of ZnO NPs (15.3 mg/L, 20.9 mg/L and 28.5 mg/L, respectively), implying that Zn^{2+} ions exhibited more severe toxicity to ammonia oxidizing bacteria than ZnO NPs. At high ZnO NPs concentration, the increased release of Zn^{2+} led eventually to the onset of inhibition of ammonia-oxidizing activity. This can also be explained by an increased production of reactive oxygen species (ROS). [21] At higher NP concentration, the increased cell surface charge and the decreased hydrophobicity may cause the worsened flocculating ability and dispersion of sludge flocs. [24]

Effect of ZnO NPs and Zn^{2+} ions on phosphate (PO_4^{3-}) uptake

In biological phosphorus removal systems, hydrolysis of polyphosphate causes soluble ortho-phosphorus (SOP) release in the anaerobic stage, which is accompanied with polyhydroxyalkanoaes (PHA) synthesis and glycogen consumption. [30] Therefore, biological phosphorus removal relies largely on the anaerobic or low-DO conditions for the transformation of intracellular PHA and glycogen. Besides biological removal, phosphorus can also be removed by coagulation and precipitation using polycations.

Low PO_4^{3-} removal efficiencies were expected in the present study due to the lack of anaerobic and anoxic conditions. However, it can be seen from Figure 5A that prior to addition of ZnO NPs, the PO_4^{3-} removal efficiency was 24.1%. However, a marked ($p < 0.05$) decrease (17.9%, 11.8% and 4.0%, respectively) was observed when activated sludge was exposed to 1.0, 10 and 50 mg ZnO NPs L^{-1}, respectively. This result showed that ZnO NPs inhibited uptake for cell synthesis. Furthermore, coagulation with Zn^{2+} ions was not observed probably due to the small amount of Zn^{2+} released. Similar results were found for the zinc salt treatment (Figure 5B). This finding is comparable with Tan et al. [26] who showed that PO_4^{3-} removal efficiency significantly decreased

to 34.3% compared to the control (47.5%), during exposure to 1 mg/L ZnO NPs. Our data therefore showed that problems in nitrogen and phosphorus removal will occur in the waste activated sludge at concentration of 1 mg/L.

Accumulation of ZnO NPs and zinc ions onto activated sludge

Activated sludge biomass from biological wastewater treatment processes is able to remove heavy metals from wastewater, and biosorption plays an important role in heavy metal recovery. [31, 32] More recently, ZnO NPs have been observed to bind onto waste activated sludge in SBR processes, [16] in MBR processes [26] and in anaerobic digestion. [33] Different partitioning mechanisms of engineered NPs to biosolids have been identified including binding to extracellular polymers or cell surface, active cellular uptake, entrapment into flocs and diffusion into biofilms [4]. In the present study, a gradual increase of Zn in biosolids was observed for both ZnO NPs and Zn^{2+} ions treatment (Figure 6). The zinc levels were respectively 2.3, 6.3, and 13.9 mg/g MLSS at 1.0, 10 and 50 mg/L ZnO NP exposure after 5 h exposure. These Zn loadings were 1.34, 2.97 and 6.74 mg/g MLSS in the $ZnSO_4$ treatment. At 50 mg/L exposure, a mass balance on Zn revealed that 88% of Zn from ZnO NPs ended up in biosolids and 12% in the effluent. For $ZnSO_4$, the mass balance was 68% onto biosolids and 32% in effluent.

By comparison, Musee et al. [17] investigated the fate and behaviour of ZnO NPs in a simulated WWTP over 240 hours and reported a mean Zn concentration of 54 mg/g MLSS and maximum Zn concentration of 112 mg/g MLSS in the sludge. This finding reinforces the results of previous studies [34, 35] which indicated that engineering ZnO NPs showed strong affinity to the sewage sludge rather than dissolution in the treatment effluent. The primary mechanism of NP removal from wastewater is believed to depend upon biosorption onto biomass.

Our finding also showed that ZnO NPs have greater potential to be adsorbed onto biosolids compared to Zn^{2+} ions. Furthermore, this biosorption capacity increased with the concentration of ZnO NPs. This result is in good agreement with Lombi et al. [33] who investigated the fate of ZnO NPs during anaerobic digestion of wastewater and reported that the partition coefficient (K_d) of Zn was smaller in the salt treatment (637 $L.kg^{-1}$) than for the ZnO NP treatments (915-1258 $L.kg^{-1}$). Their results indicate that ZnO NPs have greater potential to be adsorbed onto anaerobic sludge than Zn^{2+} ions, and that Zn derived from ZnO NPs was not partitioning in larger measure in the solution phase when compared to the Zn^{2+} salt. In addition, these observations also support the hypothesis that different mechanisms might govern the removal of ZnO NPs and Zn^{2+} ions from wastewater. As for ZnO NPs, the attenuation of the ZnO NP concentration in the solution phase is most likely due to precipitation of Zn species and ZnO NP adsorption onto the biomass. In contrast, zinc salt quickly undergo dissolution followed by complexation and precipitation.

Adsorption of ZnO NPs and Zn^{2+} ions onto activated sludge

Engineered NPs can form aggregates in the wastewater sludge through agglomeration, which involves the adherence of single or cluster of particles into larger masses due to attractive forces or chemical or mechanical binding. [11] In the present study, the morphological changes in activated sludge induced by the aggregated ZnO NPs and irreversibly agglomerated Zn^{2+} were observed by SEM (Figure 7A-7C). The SEM images clearly showed that there were large numbers of accumulated ZnO NPs on the surface of sludge after 5 h exposure. SEM images revealed differences in detrimental effect between ZnO NPs and zinc ions. Although these extent of damage cannot be accurately quantified based on our SEM analyses, the ZnO NPs appeared to have formed to larger sized aggregates during the experiment. The accumulation of ZnO NPs and Zn^{2+} on the activated sludge was also confirmed through EDS profile analysis to confirm

their Zn-based composition (Figure 7D-7E). The EDS profile clearly shows a Zn peak that is absent in the sample from the control reactor.

Bacterial viability assay

Figure 8 displays the bacterial viability in the control and in the samples treated with ZnO NPs and Zn^{2+} ions at the highest concentration after 5 h exposure. A large number of fluorescent green cells are evident in the control system (Figure 8A). Compared to the control, the density of dead cells significantly increased after the exposure of the activated sludge to 50 mg/L of ZnO NPs, indicating a great loss in the cell viability (Figure 8B). This can be due to the adsorption of NPs onto the sludge as well as the increase of dissolved Zn^{2+} content and inhibition of cell activity after exposure to 50 mg/L ZnO NPs. This phenomenon was even greater for the sludge exposed to Zn^{2+} ions (Figure 8C). The structure of the activated sludge became loose with numerous small aggregates of ZnO NPs which may result in dispersed flocs. This finding is in agreement with previous studies [24, 36] which revealed that higher concentrations of ZnO NPs exhibited inhibitory effects on the activity of activated sludge microorganisms. In addition, after 5 h exposure to ZnO NPs and Zn ions at a high concentration of 50 mg/L, the live/dead ratio exhibited a decreasing trend (2.45 and 2.26 for ZnO NPs and Zn^{2+} treatment, respectively), compared to control (2.64) (Supplementary Figure S2). This finding further confirms that the accumulated ZnO NPs on the surface of activated sludge was likely to create a stressful environment for microorganisms, thereby reducing the activity of the activated sludge. This was also supported by the significant reductions in various contaminant removal efficiencies observed during exposure to ZnO NPs and zinc ions at higher concentrations in this study.

It has been reported that the toxicity of ZnO NPs to activated sludge would be mainly due to the release of soluble Zn^{2+} ions. [16, 26] However, in the present study, only 5.6 mg Zn^{2+} $.L^{-1}$ was released from 50 mg/L ZnO NPs (Figure 2A) and it is therefore believe that biosorption of NPs onto activated sludge played a major role in inhibition mechanism as shown by the high adsorption capacity and bacterial viability analysis. In comparison, Hou et al. [37] and Li et al. [18] investigated the kinetics of Zn^{2+} released from ZnO NPs of 50 mg/L, and reported Zn level of 4.9 mg/L and 7.1 mg/L, respectively after 24 h exposure. This discrepancy might be attributable to the difference of size and surface area of investigated ZnO NPs, which in turn may lead to the toxicity induced by NPs.

Previous studies have reported that the production of extracellular polymeric substances (EPS) could strongly increase the toxicity resistance of activated sludge by preventing direct contact between zinc ions and bacteria. [26, 36] However, once the concentration of metal ions increased, the protective capacity of EPS deteriorated, due to the loose structure under high toxicity. [38] This explains the observation of increased inhibition of activated sludge at higher concentrations of ZnO NPs in the present study. The toxicity of ZnO NPs to bacteria can also be attributed to the changes in sludge properties. [24] At low concentrations of NPs, the dissolved Zn^{2+} ions from ZnO NPs could function as bridges between the functional groups on the surface of bacteria, helping to aggregate microbes and promoting bioflocculation. However, under exposure to higher concentrations of ZnO NPs, cell surface charge increases, weakening the attraction between EPS and cations, resulting in the reduction of the flocculating ability of activated sludge.

Conclusions

In this study, the fate and behaviour of ZnO NPs and zinc ions in the waste activated sludge process were investigated in SBR. The results indicate that biological wastewater treatment

plants have great potential to remove ZnO NPs from wastewater. ZnO NPs were efficiently retained by activated sludge, and exhibited greater biosorption capacity and strong affinity to the sewage sludge, compared to Zn^{2+} ions. The short-term exposure to ZnO NPs at 1 mg/L showed some effects on COD removal, ammonia removal and phosphorus uptake. Exposure to 10 mg/L and 50 mg/L significantly inhibited the biological wastewater treatment process. Compared to ZnO NPs, Zn^{2+} ions exhibited more severe toxicity towards activated sludge at high concentrations due to a better dissolution of Zn^{2+} from $ZnSO_4$. The results of bacterial integrity analysis showed that accumulated ZnO NPs on the surface of activated sludge created a stressful environment for microorganisms, as shown by a decreasing live/dead ratio, thereby reducing the activity of activated sludge.

Acknowledgements

The authors would like to express sincere thanks to the Singapore Economic Development Board and the Environment & Water Industry Programme.

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FIGURE CAPTIONS

Figure 1 TEM image of ZnO NPs in the nutrient solution under different magnification: (A) 0.5 μm; (B) 100 nm; (C): 50 nm

Figure 2 Kinetics of Zn^{2+} released from a) ZnO NPs at the concentrations of 1.0, 10 and 50 mg/L; and b) $ZnSO_4 \cdot 7H_2O$ at the concentrations of 3.54, 35.4 and 177 mg/L. Error bars represent standard deviations of triplicate measurement. The error bars were omitted when smaller than the marker.

Figure 3 COD concentrations in the effluent of a) ZnO NP treatment; and b) Zn^{2+} ions treatment

Figure 4 NH_4-N concentrations in the effluent of a) ZnO NP treatment; and b) Zn ions treatment. Error bars represent standard deviations of triplicate measurement

Figure 5 Phosphate concentrations in the effluent exposed to a) ZnO NPs; and b) Zn^{2+} ions. Error bars represent standard deviations of triplicate measurement

Figure 6 Zinc levels in the biosolids for a) ZnO NP treatment; and b) Zn^{2+} treatment. Error bars represent standard deviations of triplicate measurement

Figure 7 SEM images of activated sludge after ZnO NPs and Zn^{2+} ions exposure at the concentration of 50 mg/L after 5 h. a) Sludge in the control; b) Sludge in the treatment exposed to ZnO NPs; and c) Sludge in the treatment exposed to Zn^{2+} ions; d) EDS spectra for a); e) EDS spectra for b); and f) EDS spectra for c)

Figure 8 Bacterial viability in a) control treatment; b) in the activated sludge exposed to ZnO NPs; and c) in the activated sludge exposed to zinc salt at the concentration of 50 mg L^{-1} after 5 h exposure

Supplementary Fig S1. Size distribution of ZnO NPs. The size range determined using TEM as 15-47 nm with a mean size of 33± 8 nm (n=107).

Supplementary Fig S2. Live/dead ratio after 5 hours exposure of ZnO NPs and Zn ions.

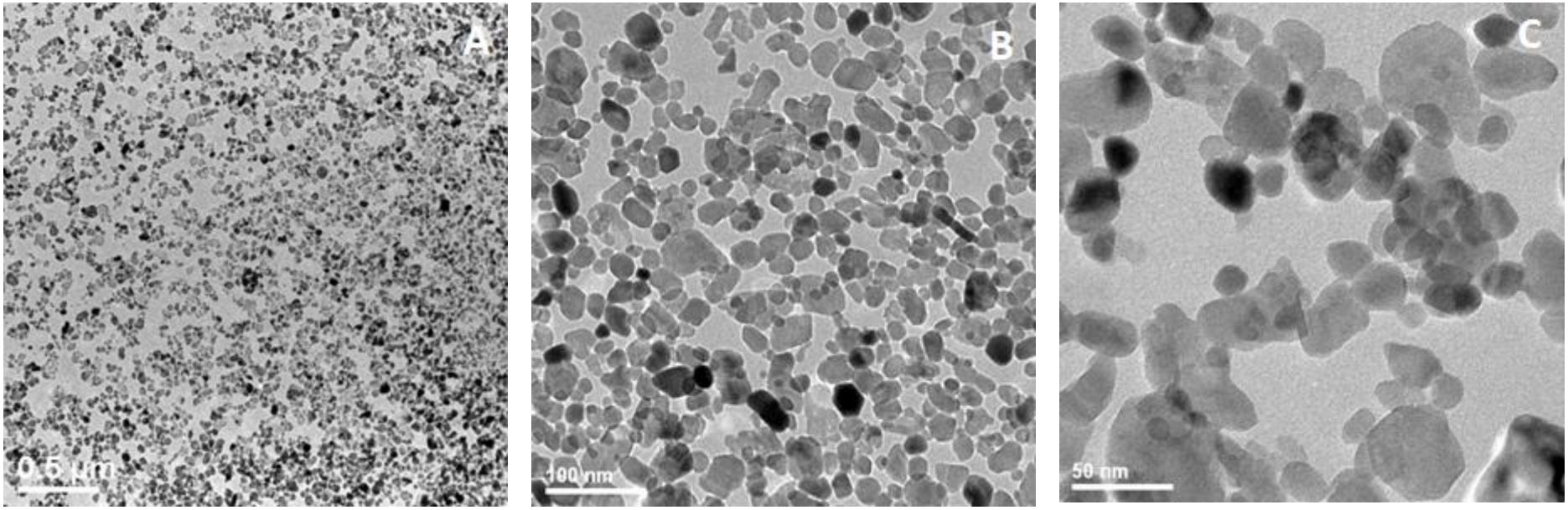


Fig. 1

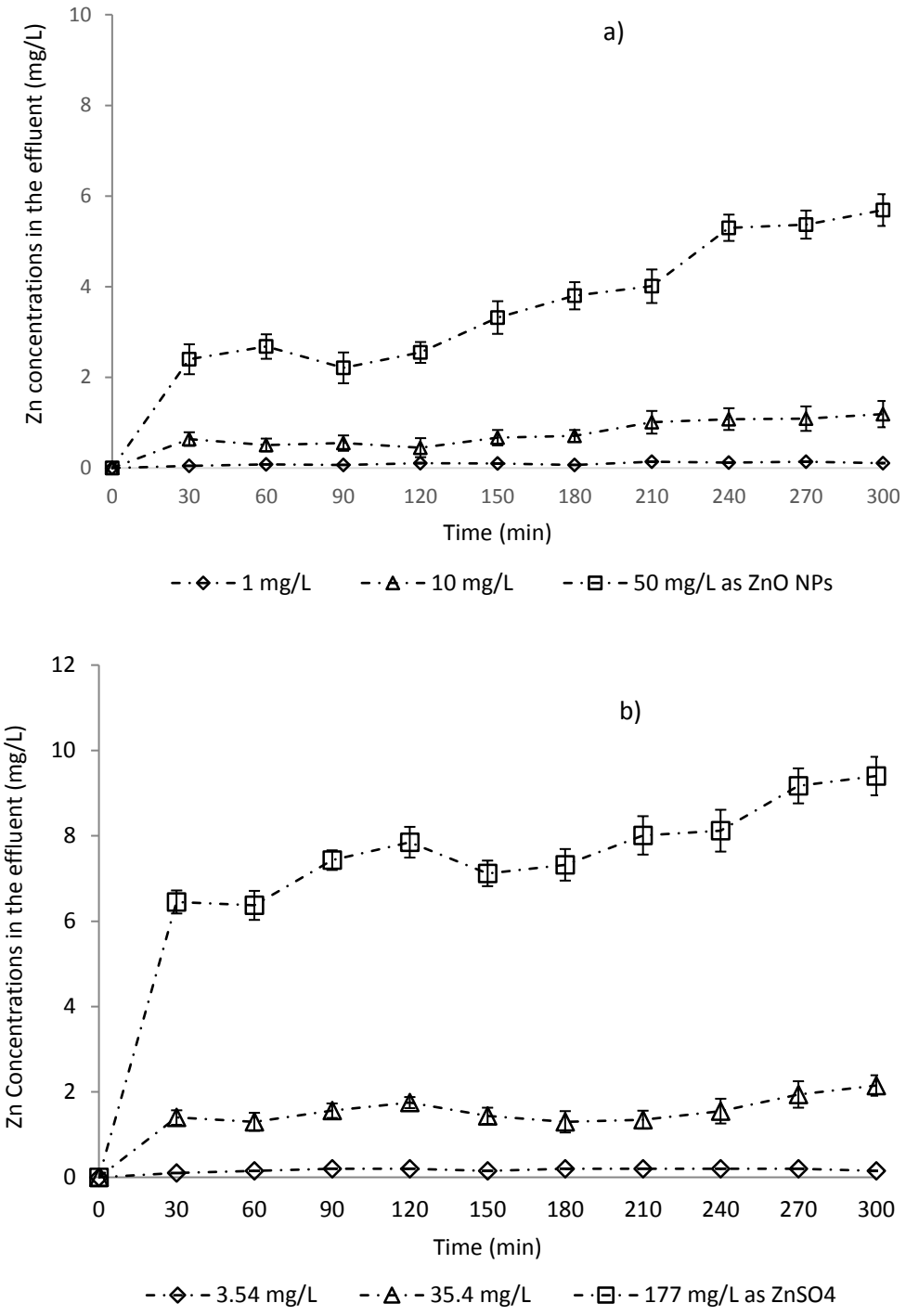
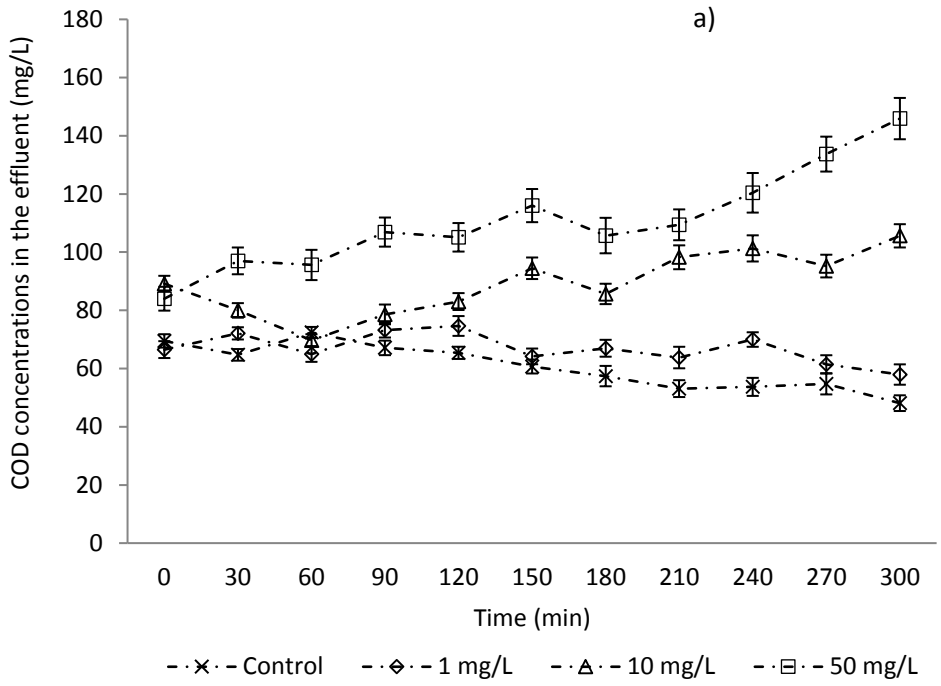

a)
Zn concentrations in the effluent (mg/L)
10
8
6
4
2
0
0
30
60
90
120
150
180
210
240
270
300
Time (min)
1 mg/L
10 mg/L
50 mg/L as ZnO NPs
b)
Zn Concentrations in the effluent (mg/L)
12
10
8
6
4
2
0
0
30
60
90
120
150
180
210
240
270
300
Time (min)
3.54 mg/L
35.4 mg/L
177 mg/L as ZnSO4

Fig. 2


a)
COD concentrations in the effluent (mg/L)
180
160
140
120
100
80
60
40
20
0
0 30 60 90 120 150 180 210 240 270 300
Time (min)
Control
1 mg/L
10 mg/L
50 mg/L

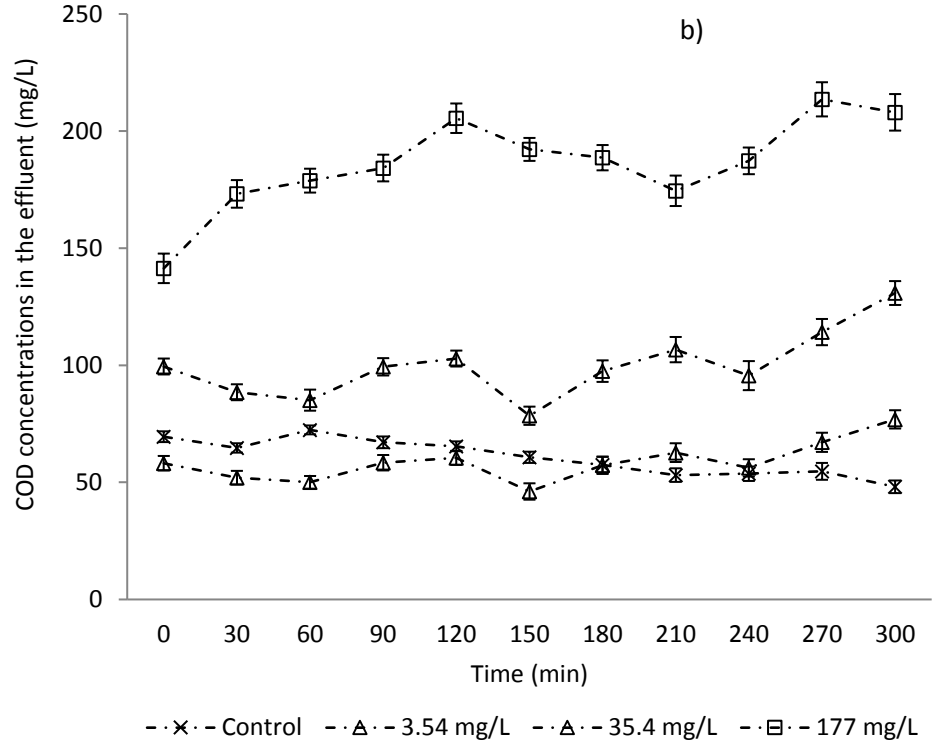
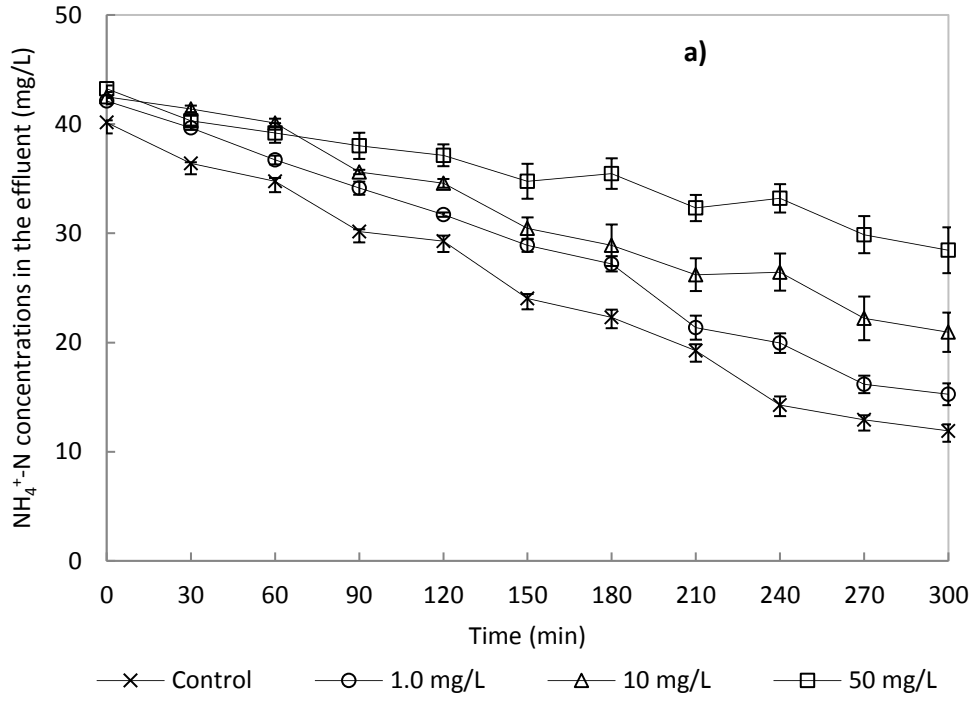

b)
COD concentrations in the effluent (mg/L)
250
200
150
100
50
0
0 30 60 90 120 150 180 210 240 270 300
Time (min)
Control
3.54 mg/L
35.4 mg/L
177 mg/L

Fig. 3


a)
50
40
30
20
10
0
NH_4^+-N concentrations in the effluent (mg/L)
0
30
60
90
120
150
180
210
240
270
300
Time (min)
Control
1.0 mg/L
10 mg/L
50 mg/L

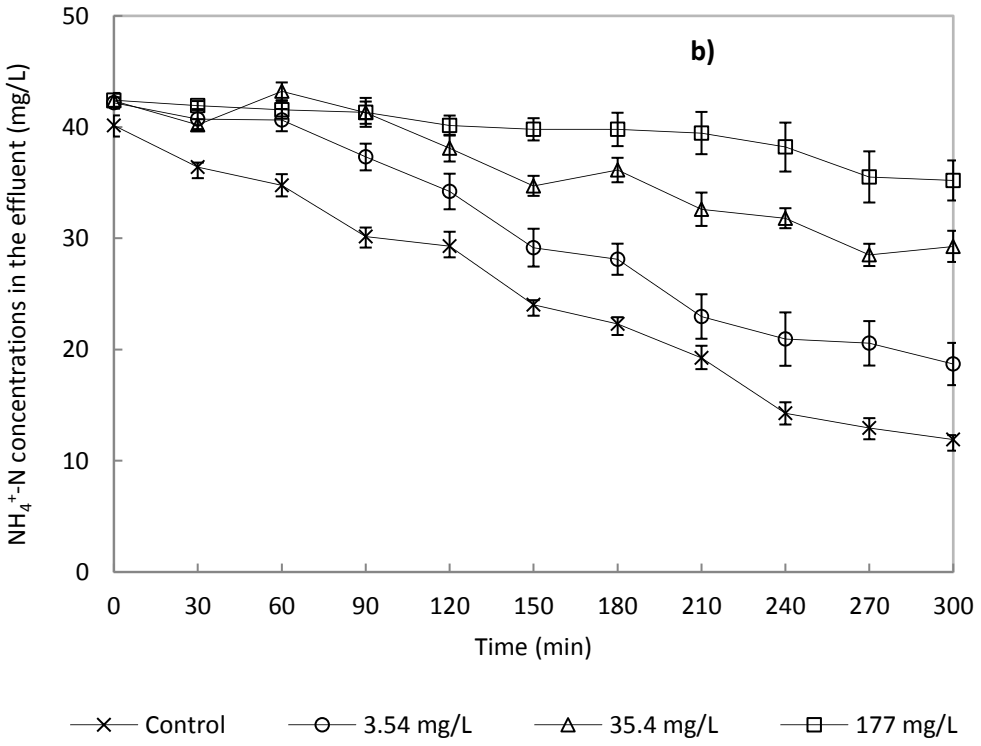
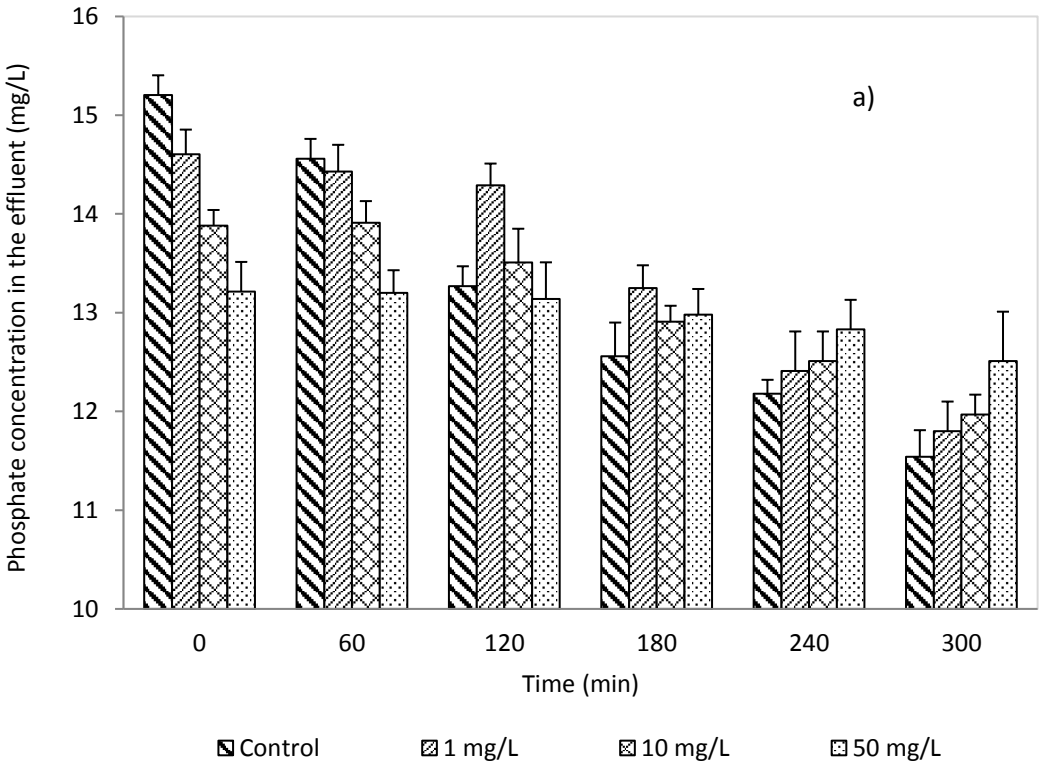

b)
50
40
30
20
10
0
NH_4^+-N concentrations in the effluent (mg/L)
0
30
60
90
120
150
180
210
240
270
300
Time (min)
Control
3.54 mg/L
35.4 mg/L
177 mg/L

Fig. 4


a)
Phosphate concentration in the effluent (mg/L)
16
15
14
13
12
11
10
0
60
120
180
240
300
Time (min)
Control
1 mg/L
10 mg/L
50 mg/L

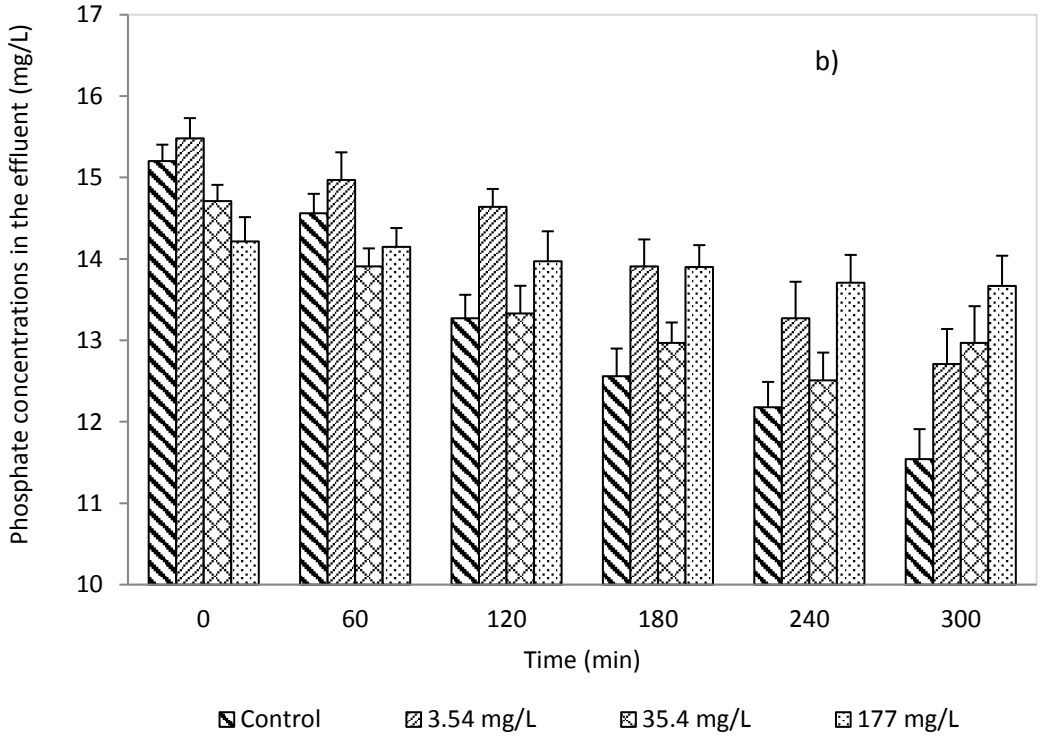

b)
Phosphate concentrations in the effluent (mg/L)
17
16
15
14
13
12
11
10
0
60
120
180
240
300
Time (min)
Control
3.54 mg/L
35.4 mg/L
177 mg/L

Fig. 5

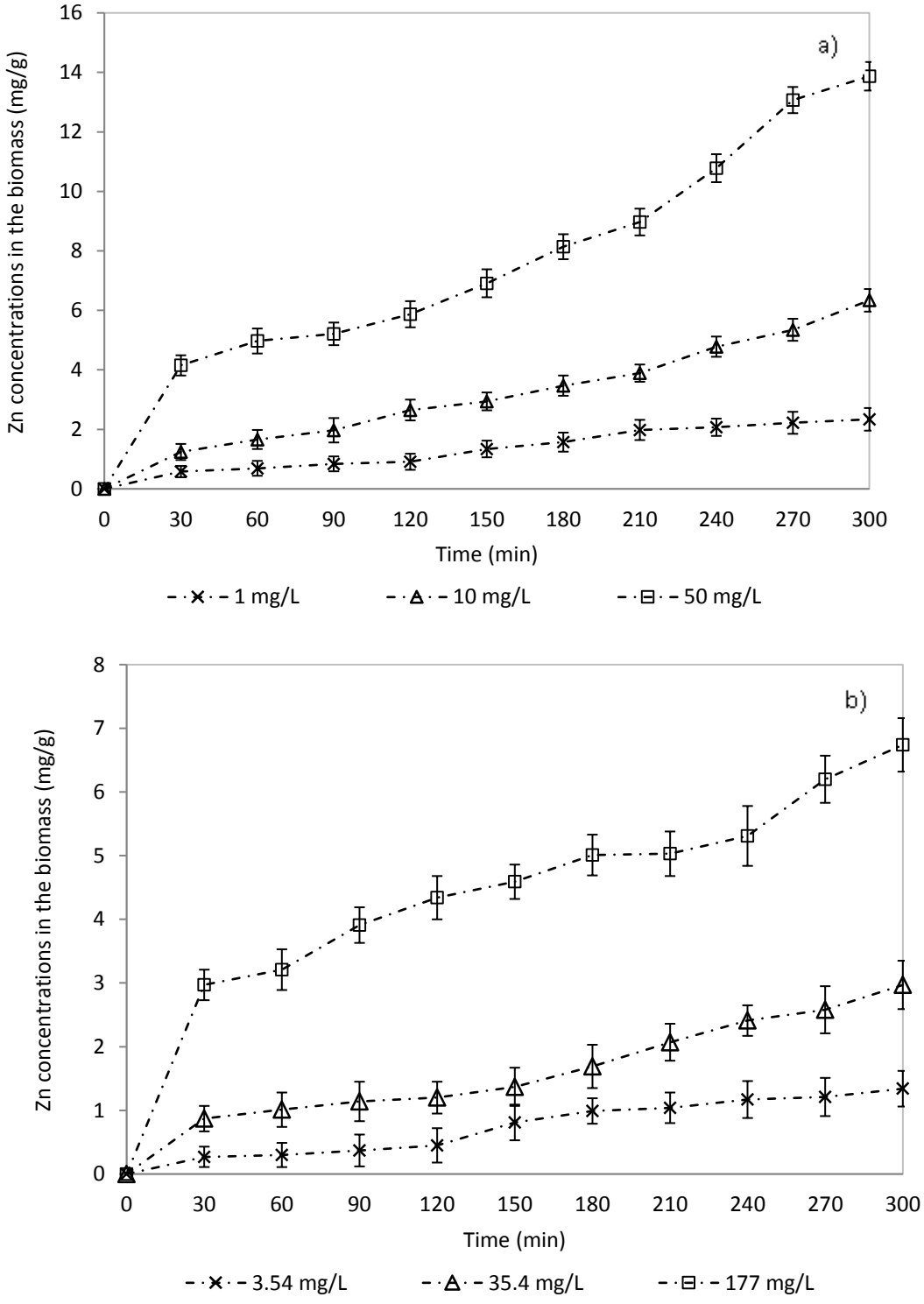

a)
Zn concentrations in the biomass (mg/g)
16
14
12
10
8
6
4
2
0
0
30
60
90
120
150
180
210
240
270
300
Time (min)
1 mg/L
10 mg/L
50 mg/L
b)
Zn concentrations in the biomass (mg/g)
8
7
6
5
4
3
2
1
0
0
30
60
90
120
150
180
210
240
270
300
Time (min)
3.54 mg/L
35.4 mg/L
177 mg/L

Fig. 6

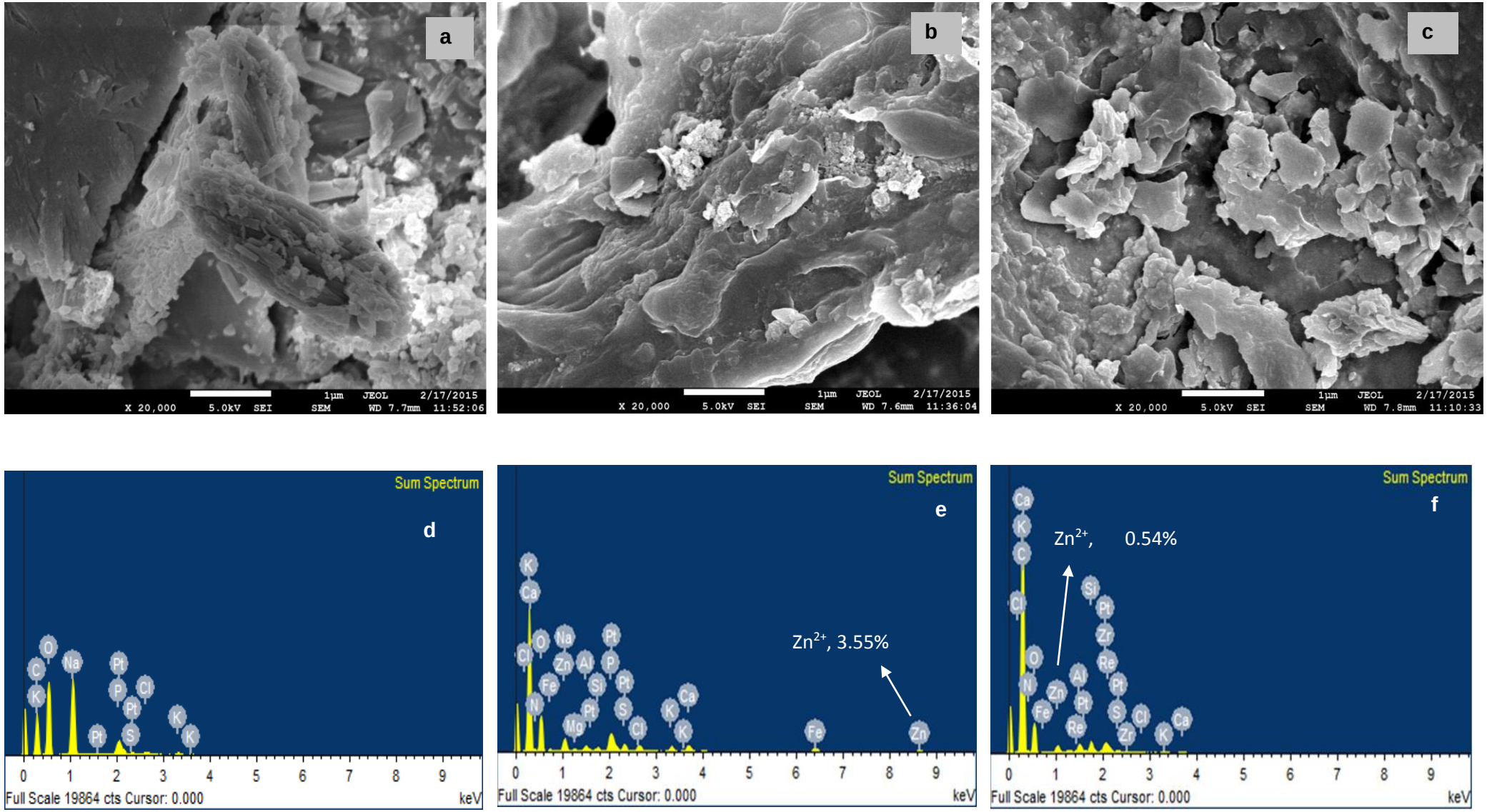

a
1µm JEOL 2/17/2015
X 20,000 5.0kV SEI SEM WD 7.7mm 11:52:06
b
1µm JEOL 2/17/2015
X 20,000 5.0kV SEI SEM WD 7.6mm 11:36:04
c
1µm JEOL 2/17/2015
X 20,000 5.0kV SEI SEM WD 7.8mm 11:10:33
Sum Spectrum
d
Full Scale 19864 cts Cursor: 0.000
keV
Sum Spectrum
e
Zn2+, 3.55%
Full Scale 19864 cts Cursor: 0.000
keV
Sum Spectrum
f
Zn2+, 0.54%
Full Scale 19864 cts Cursor: 0.000
keV

Fig. 7

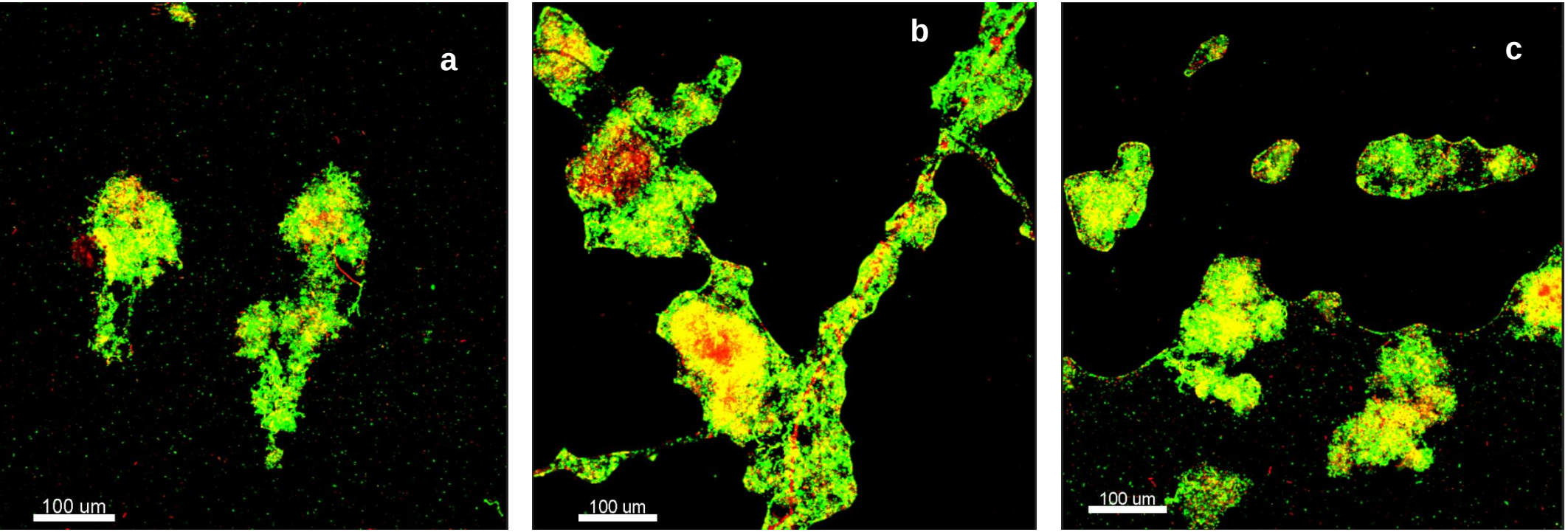


Fig. 8

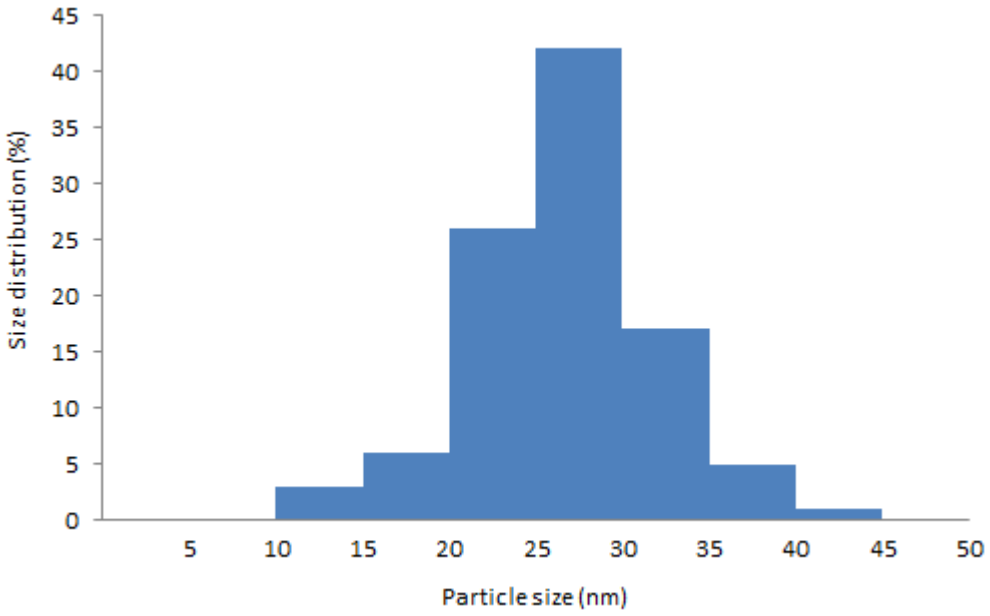


Fig S1. Size distribution of ZnO NPs. The size range determined using TEM as 15-47 nm with a mean size of 33± 8 nm (n=107).

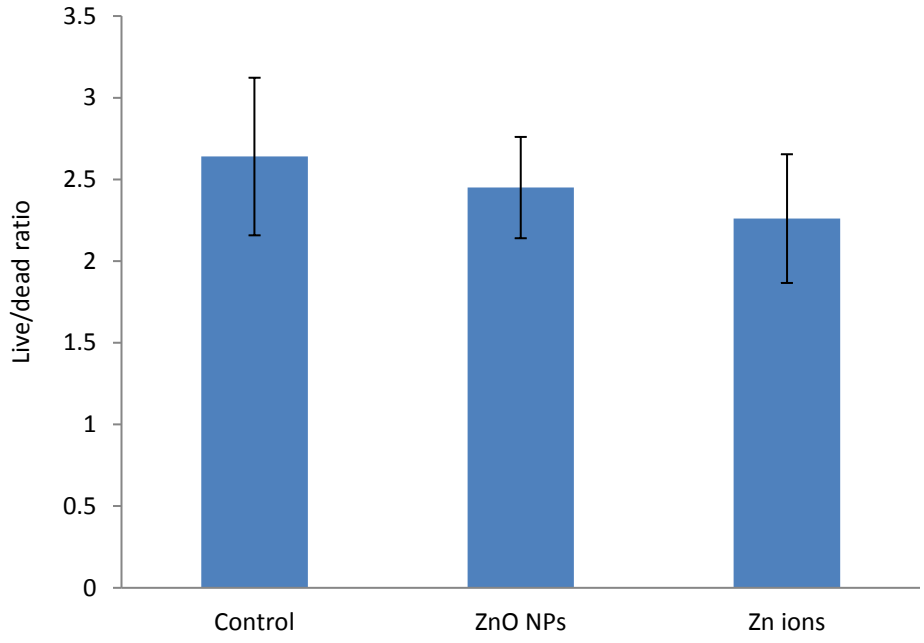


Fig S2. Live/dead ratio after 5 hours exposure of ZnO NPs and Zn ions.