Comparison and Distribution of Copper Oxide Nanoparticles and Copper Ions in Activated Sludge Reactors.

DONGQING ZHANG[1], ANTOINE P. TRZCINSKI[2*], HYUN-SUK OH[3], EVELYN CHEW[1], SOON KEAT TAN[1], WUN JERN NG[1], YU LIU[1].

[1]*Advanced Environmental Biotechnology Centre, Nanyang Environment and Water Research Institute, 1 Cleantech loop, #06-10, Singapore 637141.*

[2]*School of Civil Engineering & Surveying, Faculty of Health, Engineering and Sciences, University of Southern Queensland, 4350 Australia.*

[3]*Singapore Membrane Technology Centre, Nanyang Environment and Water Research Institute, 1 Cleantech loop, #06-10, Singapore 637141.*

*Address correspondence to Dr Antoine TRZCINSKI, School of Civil Engineering & Surveying, Faculty of Health, Engineering and Sciences, University of Southern Queensland, 4350 Australia, Telephone number: +61 7 4631 1617;

Email: antoine.trzcinski@usq.edu.au,

Abstract

Copper oxide nanoparticles (CuO 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 Copper Oxide NPs at concentrations of 0.1, 1, 10 and 50 mg/L and compare it with its ionic counterpart (as $CuSO_4$). It was found that 0.1 mg/L CuO NPs had negligible effects on Chemical Oxygen Demand (COD) and ammonia removal. However, the presence of 1, 10 and 50 mg/L CuO NPs decreased COD removal from 78.7% to 77%, 52.1% and 39.2%, respectively ($p<0.05$). The corresponding effluent ammonium (NH_4-N) concentration increased from 14.9 mg/L to 18, 25.1 and 30.8 mg/L, respectively. Under equal Cu concentration, copper ions were more toxic towards microorganisms compared to CuO NPs. CuO NPs were removed effectively (72-93.2%) from wastewater due to a greater biosorption capacity onto activated sludge, compared to the copper ions (55.1%-83.4%). The SEM images clearly showed the accumulation and adsorption of CuO NPs onto activated sludge. The decrease in Live/dead ratio after 5 h exposure of CuO NPs and Cu^{2+} indicated the loss of cell viability in sludge flocs.

Keywords: CuO nanoparticles; copper 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. [1-3] As one of the most important engineered applications, copper oxide nanoparticles (CuO NPs) exhibit optical, electrical and catalytic properties, and have been used intensively in electronics,

ceramics, chemical sensors, polymers inks, metallic and coating. [4-6] Particularly, CuO NPs are commonly generated in large amounts during wafer chemomechanical polishing operations, which is a major source of wastewater in semiconductor manufacturing. [7] The increasing use of CuO NPs in industry and consumer products raises the concerns about the environmental risks due to their novel physical and chemical properties. Therefore, it is imperative to understand the environmental impact of CuO NPs.

Results from material flow analyses suggest that a major fraction of the NPs in commercial products will eventually enter municipal or industrial wastewaters, and subsequently reach wastewater treatment plants (WWTPs). [8, 9] WWTPs are considered as the last barriers prior to their environmental release. [10] Therefore, efficient removal of engineered NPs from wastewater is particularly important in view of their increasing evidence for their ecotoxicity. [11] Furthermore, their toxicity to some microorganisms within the biological systems of WWTPs is of particular concern, since the inhibition and loss of certain bacterial species involved could be detrimental to biological treatment performance. [12] Previous study by Otero-González et al. [13] indicated that the extended exposure to even relatively low concentration (1.4 mg/L) of CuO NPs had a markedly negative effect on the performance of methanogenesis in upflow anaerobic sludge blanket (UASB) reactor. In another recently study, 50% inhibition of CH_4 production was also observed during anaerobic digestion processes in the presence of 11 mg Cu L^{-1} of CuO NPs over a 14-d period. [14]

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 the properties (size, charge density), chemical composition of media (pH, organics, ionic strength), test conditions, and organisms evaluated. [10] CuO NPs and Cu^{2+} ions were reported to show different toxicity to some microbes. [15, 16] In a recent study of the toxic effects of CuO NPs, bulk CuO and $CuSO_4$ on *Tetrahymena thermophila*, Mortimer et al. [15] indicated that the most toxic Cu compound was

$CuSO_4$, which was approximately 120 times more toxic than CuO NPs and 1500 times more toxic than bulk CuO. The different toxicity of Cu compounds has also been reported in a study of Heinlaan et al. [16] where the EC_{50} values for bulk CuO, CuO NPs and $CuSO_4$ were 3811, 79, 1.6 mg/L (*Vibrio fischer*); 165, 3.2, 0.17 mg/L (*Daphnia magna*); and 95, 2.1, 0.11 mg/L (*Thamncephalus platyurus*), respectively. However, Aruoja et al. [17] investigated the toxicities of ZnO, TiO_2 and CuO NPs to mircoalgae *Pseudokirchneriella subcapitata* and reported that the bioavailable EC50 values of CuO NPs were not significantly different from the EC50 of $CuSO_4$ (0.02 mg Cu/L).

There is a lack of information on the behaviour of CuO NPs in WWTPs and the effects of CuO NPs on the treatment performance in terms of organic removal and nitrification. [12, 13] In particular, a detailed evaluation of the extent to which CuO NPs were removed, characteristics of CuO NPs in suspension and/or sludge, and a comparison of the above with ionic salts, is currently not available. [10] Most authors have investigated specific microorganisms or activated sludge fed with synthetic wastewater. Studies with real wastewater are still scarce, but important because interactions with natural organic matter in real wastewater may result in different behaviour of CuO NPs. For instance, Cu ions can generate complex with humic acids due to their carboxylic and phenolic groups or precipitate as insoluble copper hydroxide.

Therefore, the objectives of this study were (a) to compare the short term effects and fate of CuO NPs and Cu^{2+} in a laboratory scale waste activated sludge process fed with real wastewater; (b) to investigate the effects of 0.1, 1, 10 and 50 mg/L CuO NPs on COD and nitrogen removals; (c) to determine the accumulation of Cu 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 CuO NPs and Cu^{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 and wastewater

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.

CuO NPs characterization

The CuO NPs were purchased from Sigma-Aldrich (Singapore) with average particles size of 40±5 nm. CuO NPs stock solutions (100 mg/L) were prepared by adding dry particles into Milli-Q (pH=6.8±0.2), and then the suspensions were sonicated (30°C, 100 W, 40 kHz) for 30 min and shaken for 2 h to increase their dispersion. *Zeta* potential of CuO NPs in the suspensions were measured using a Nanosizer (Malvern Instruments Ltd., UK). The morphology of the CuO 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 the use the suspension of CuO NPs.

125

126 ***Sequencing batch reactors (SBR)***

127

128 SBRs were designed to simulate a full-scale operation of aeration and secondary clarification as
129 described by Hou et al. [18] The SBRs (0.5 L) were seeded with return nitrifying activated sludge
130 from Changi Water Reclamation Plant (Singapore) adjusted to a mixed liquor suspended solids
131 (MLSS) concentration of 3 g/L. The hydraulic retention time (HRT) was 12 hours, while the
132 sludge retention time (SRT) was 15 days. The steady state was established through monitoring
133 the chemical oxygen demand (COD) and ammonium. The SBRs were operated under anoxic-
134 aerobic conditions and each cycle had a duration of 8 h, including 1 h feeding, 1 h of anoxic
135 period, 3 hours of aeration, settling for 2 h and effluent withdrawal for 1 h. After each cycle,
136 supernatants following settling were replaced with primary clarifier effluent from Ulu Pandan
137 Water Reclamation Plant to start the next cycle. The general parameters, such as pH, dissolved
138 oxygen, and temperature were monitored and automatically recorded using a data logger. Both
139 SBRs were run at a temperature of 24-26°C.
140 After 15 days of stabilisation period, four SBRs were spiked with CuO NPs at the concentrations
141 of 0.1, 1, 10, and 50 mg CuO/L, respectively and three SBRs were spiked with corresponding
142 ionic salt (in the form of $CuSO_4$) at concentration of 0.2, 2.0, 20, and 100 mg/L $CuSO_4$/L such
143 that both sets of SBR contained exactly 0.08, 0.8, 8.0 and 40.0 mg Cu^{2+}/L, respectively. One
144 SBR was employed as control with no Copper addition. Each condition was operated for one
145 month and steady state data were collected over three cycles to determine average and standard
146 deviation.

147

148 ***Analytical methods***

149

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 measurement of MLSS, mixed liquor volatile suspended solids (MLVSS), chemical oxygen demand (COD), ammonium (NH_4^+-N), and phosphate (PO_4^{3-}) was in accordance with the Standard Methods. [19] All chemical tests were done in triplicate.

The Cu levels in both liquid sample and biosolids were determined as described by microwave plasma – Atomic Emission Spectroscopy (MP-AES). [13] Briefly, 10 mL collected samples were first centrifuged for 10 min at 10,000 rpm prior to metal analysis (Eppendorf 5810R). Then the supernatant (2 mL) were collected and mixed with 2 mL of HNO_3 (69%, Sigma-Aldrich) and shaken overnight at 30±2°C to ensure complete Cu dissolution. Thereafter, Cu concentrations in liquid samples were determined by MP-AES (4100, Agilent Technologies) in triplicate. Cu level in biosolids was measured after digestion in an Anton Paar Microwave Reaction System (Multiwave 3000, Alpha Analytical USA) following EPA method 3051A. [13] All chemical tests were done at least in duplicates.

Bacterial viability assay

The impact on bacteria integrity in the presence of CuO NPs and copper salt were assessed using a LIVE/DEAD *Bac*light bacterial viability kit (Molecular Probes, USA). Viable and dead cells were detected by a green fluorescent nucleic acid stain, SYTO 9, which generally labels all bacteria (live and dead) with a green fluorescence, and a red fluorochrome, propidium iodide (PI), which stains only bacteria with damaged membranes due to its membrane impermeability. At the end of the experiment, 1 mL of the sludge suspension was stained with 1.5 μL of SYTO9 and 1.5 μL of PI for 15 min in the dark at room temperature. The stained samples was covered with

cover slip and visualized using Nikon A1R confocal laser scanning microscope (CLSM) system attached to an upright ECLIPSE 90i machine with a 40× objective lens (Nikon, Tokyo, Japan). All images were acquired at a scale of 79.55 μm × 79.55 μm with 5.11 μm of confocal slice. The images were further analysed by Imaris software (Bitplane AG, Zurich, Switzerland) to calculate live/dead ratio.

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

Samples were investigated using TEM and SEM. In the first case TEM grids were prepared by placing a drop of suspension (mixed liquor or supernatant) on a holey carbon grid and drawing the suspension through the TEM grid using a paper tissue. The TEM grids were washed afterwards in a drop of distilled water to remove the dissolved compounds. [20] The TEM was operated at 200 kV to detect and characterize aggregation state of NPs in the solution.
To prepare SEM image, mixed liquor was first washed 3 times with 0.1 M phosphate buffer solution (PBS) (pH 7.7) and fixed in 0.1 M phosphate buffer (7.4) containing 2.5% glutaraldehyde at 4 °C for 4 h. The dried samples were coated with platinum before SEM analysis according to Zheng et al. (2011). The elemental analysis of the particles was carried out using an energy-dispersive X-ray spectroscope (EDS).

Statistical analysis

The results are presented as average± standard deviation for each concentration. Tests to determine statistical differences between treatments were carried out by comparing the critical value through ANOVA one-way analysis of variance (SPSS Statistics V17.0). Comparisons were considered significantly different at $p < 0.05$.

Results and discussion

Characterization of CuO NPs

Figure 1 shows the TEM image of CuO NPs in deionized water under different magnifications (0.5 µm, 100 nm and 50 nm). In the present study, due to their small size and huge surface area, NPs tend to aggregate or agglomerate in aqueous phase. Although the CuO NPs used in this study have a diameter size within the nanometer range, some aggregates of different sizes were formed in the solution where the particles were suspended, even after sonication. The *zeta* potential was -41.7 mV at pH= 6.8 and -35.6 mV at pH=6.4 at the beginning and end of the experiment, respectively.

Removal of CuO NPs and copper ions

The Cu levels in the biomass-free effluent spiked with CuO NPs and copper salt is shown in Figure 2A. After 5 h exposure, the concentrations of released soluble Cu^{2+} were 0.028, 0.204, 1.02 and 2.81 mg/L at the initial CuO NP concentration of 0.1, 1.0, 10 and 50 mg/L, respectively. This finding indicates that the majority of the Cu in the influent was adsorbed onto settled biomass. At the CuO NP concentrations of 0.1 and 1.0 mg/L, both supernatant and effluent Cu content were consistently low. The higher concentrations of released Cu^{2+} observed at the initial CuO NP concentrations of 10 mg/L and 50 mg/L can be attributed to the increased sludge surface charge and the decreased hydrophobicity resulting in more Cu^{2+} ions released from CuO NPs. [21] Furthermore, the Cu concentrations in copper salt treatment were 3.2, 3.1, 4.9 and 5.9 fold higher than in the corresponding CuO NPs treatment (Fig. 2B). Less Cu^{2+} was released from

NP possibly because humic acids are able to stabilize nanoparticles and retard dissolution rates. [22]

Interestingly, CuO NPs were removed more efficiently than copper salt in this study with removal efficiencies ranging from 72% to 93.2% for CuO NPs, while the values were 55.1% to 83.4% for Cu^{2+} ions treatment, suggesting that large fraction of CuO NPs was removed from the wastewater. These observations also support the hypothesis that the mechanisms governing the removal of CuO NPs and ionic copper are different. As for copper salt, it is highly possible that the majority of the added copper salt may quickly undergo a transformation due to their dissolution followed by complexion or precipitation. [10, 23] Furthermore, depending on the wastewater characteristics, copper can also be removed by coagulation or ion exchange in wastewaters. [24, 25] In contrast, the attenuation of the CuO NP concentration in the liquid is most likely due to aggregation, settling and biosorption onto the biomass. [12, 26, 27]

Effect of CuO NPs and copper ions on COD removal

Prior to addition of CuO NPs, the COD concentration in the effluent was around 130 mg/L which corresponds to a COD removal efficiency of 78.7% (Fig. 3). The presence of CuO NPs, however, influenced the COD removal efficiencies, which slightly decreased to 77% ($p<0.05$) at CuO NP concentrations of 1 mg/L, respectively. The exposure to 10 and 50 mg/L CuO NPs further decreased COD removal efficiencies to 52.1% and 39.2%, respectively. The lower COD removals was due to the high toxicity of the released Cu^{2+} ions from CuO NPs which inhibited microorganisms. It can also be explained by the increased cell surface charge resulting in reduced hydrophobicity and floc breakage as suggested by previous studies. [28, 29] Our finding implies that 1 mg/L CuO NPs will cause some disturbance to the waste activated sludge process which was not reported previously. This finding is in disagreement with Tan et al. [29] who

revealed that both short- and long term exposure of 1.0 mg/L of ZnO NPs did not significantly impact COD removal, despite the fact that ZnO NPs may exhibit more toxic effects on specific microorganisms than CuO NPs. Chen et al. [21] investigated the influence of Cu NPs on the physical-chemical properties of activated sludge, and indicated that lower Cu NPs concentrations (5 mg/L) did not affect the sludge properties, while higher Cu NPs concentrations (30-50 mg/L) may deteriorate the physical-chemical properties of activated sludge.

When $CuSO_4$ was used, the Cu^{+2} concentration quickly increased to 4.1 mg/L after only 30 minutes and gradually increased to 16.6 mg/L after 300 minutes, which resulted in a greater toxicity. In this study, in the presence of 20 and 100 mg/L copper sulphate, COD removals were 44.8% and 7.3%, which were significantly ($p < 0.05$) lower than those (52.1% and 39.2%) in the presence of CuO NPs, showing that copper salt exhibited more severe toxicity towards microbes than CuO NPs. Moreover, the MLSS concentration decreased markedly to 1.2 g/L with 100 mg/L $CuSO_4$ (data not shown), showing that flocs were disrupted and cell lysis took place. From Figures 2 and 3, it is clear that CuO NPs is less toxic than $CuSO_4$ due to the fact that Cu ions from $CuSO_4$ dissolve more readily in water. These findings are consistent with Heinlaan et al. [16] who evaluated the eco-toxicity of ZnO NPs, CuO NPs and TiO_2 to bacteria and crustaceans, and reported that $CuSO_4$ was approximately 100-fold more toxic than nano CuO to *Vibrio fischer* with LC_{50} value of 1.6 versus 79 mg/L, and 1000-fold more toxic than nano CuO to *Daphnia magna* (0.17 versus 164.8 mg/L) and *Thamncephalus platyurus* (0.11 versus 94.5 mg/L). In this study, after the addition of 50 mg/L CuO-NPs (equivalent to 40 mg/L Cu^{+2}), the measured Zn^{2+} concentration in the effluent progressively increased to only 2.8 mg/L after 5 hours, indicating a low dissolution potential of ZnO-NPs in the system, and that the most likely cause of inhibition was therefore the adsorption of CuO NP onto bacterial cells.

Effect of CuO NPs and copper ions on ammonium removal

The effect of CuO NPs and copper ions on NH_4^+-N removal are shown in Figure 4. The NH_4^+-N removal in the presence of 0.1 (64.1%) were relatively stable with increasing exposure time and not statistically different ($p<0.05$) from the negative control at (64.8%) over a period of 5 h exposure. However, when activated sludge was exposed to 1, 10 and 50 mg/L CuO NPs, the effluent NH_4^+-N significantly ($p<0.05$) increased from 14.9 mg/L (control) to 18 mg/L, 25.1 mg/L and 30.8 mg/L, respectively, suggesting that CuO NPs at 1 mg/L could start causing some inhibition to ammonia oxidizing bacteria. At higher CuO NP concentration, the flocculating ability deteriorated due to the increased cell surface charge and the decreased hydrophobicity made the sludge flocs more dispersed, which further increased the toxicity of the CuO NPs by increasing the contact between CuO NPs and bacteria. [21] This finding also indicated that biosorption of CuO NPs onto activated sludge induced adverse effects on the diversity and activity of nitrifying microbial species. Additionally, in the present study, effluent ammonia concentration (20.7 mg/L, 29.3 mg/L and 35.2 mg/L, respectively) in the presence of $CuSO_4$ were higher than those in the presence of ZnO NPs (18 mg/L, 25.1 mg/L and 30.8 mg/L, respectively), implying that Cu^{2+} ions exhibited more severe toxicity to ammonia oxidizing bacteria than ZnO NPs.

Accumulation of CuO NPs and copper 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. [30, 31] CuO NPs and dissolved Cu^{2+} have been observed to bind on the surface of activated sludge. [32] Previous studies reported that biosorption of CuO NPs can take place in activated sludge treatment [12] and anaerobic sludge treatment exposed to synthetic wastewater. [13] Different

mechanisms of partitioning of NPs to biosolids have been identified including binding to extracellular polymers or cell surface, active cellular uptake, entrapment into flocs and diffusion into biofilms. [33] In the present study, a gradual increase in the Cu^{2+} concentrations in the biosolids was observed for both CuO NPs and copper salt treatment (Fig. 5). The copper concentrations were 2.12, 7.29, 11.1 and 29.31 mg/g MLSS at the CuO NP concentrations of 0.1, 1.0, 10 and 50 mg/L after 5 h exposure, respectively, which was 1.58, 1.51, 1.10 and 1.68 fold more than in the $CuSO_4$ treatment. At 50 mg/L exposure, a mass balance on Zn revealed that 98% of Cu from CuO NPs ended up in biosolids and 2% in the effluent. For $CuSO_4$, the mass balance was 86% onto biosolids and 14% in effluent. This finding suggests that CuO NPs have greater potential for adsorption onto biosolids compared to Cu^{2+} ions, due to its smaller particles size and larger surface area, and this biosorption capacity increased with the concentration of CuO NPs. Furthermore, the higher copper levels found in the biosolids were mainly attributed to CuO NPs, instead of the released Cu^{2+} from CuO NPs, given the fact that CuO NPs have much less Cu^{2+} release capacity, compared to copper salt. This finding also reinforces the results of previous studies [11, 34] which indicated that the primary process of NP removal from wastewater is believed to be associated with biosorption onto biomass, although NPs may undergo transformation (e.g., dissolution of metal ions from metal-based NPs). In addition, these observations also support the hypothesis that different mechanisms might govern the removal of CuO NPs and Cu^{2+} ions from wastewater. As for CuO NPs, the attenuation of the CuO NP concentration in the solution phase is most likely due to precipitation of Cu species and CuO NP adsorption onto the biomass. In contrast, copper salt quickly undergo dissolution followed by complexation and precipitation.

The morphological changes in the activated sludge induced by the accumulated CuO NPs and Cu^{2+} were observed by SEM (Fig. 6A-6C). After 5 h exposure, the SEM images clearly showed

the accumulation and adsorption of CuO NPs onto activated sludge. Such observation corroborates previous study assessing the effect of CuO NPs on physicochemical stability of activated sludge flocs. [12] SEM images revealed differences in damage extent between CuO NPs and copper salt. Although these damage extent cannot be accurately quantified based on our SEM analyses, the ionic copper appeared to have transformed to larger size aggregates during the experiment. The accumulation of CuO NPs and Cu^{2+} on activated sludge was also confirmed using EDS profile analysis to confirm their Cu-based composition (Fig. 6D-6E). The EDS profile clearly demonstrates a Cu peak that is absent in the sample from the control reactor.

Bacterial viability assay

Figure 7 displays the bacterial viability in the control and in the activated sludge exposed to CuO NPs and copper salt for 5 h. Compared to the control (Fig. 7A), the density of the dead cells increased after the exposure of the activated sludge to 50 mg/L of CuO NPs (Fig. 7B) or 100 mg/L Cu^{2+} ions (Fig. 7C), indicating a loss in the cell viability. The structure of the activated sludge became loose with numerous small aggregates of bacterial cells which may result in dispersed flocs. This can be due to the adsorption of NPs onto the sludge and inhibition of cell activity after exposure to 50 mg/L ZnO NPs. This was supported by the significant reduction in contaminant removal observed under the exposure to CuO NPs and copper ions at higher concentrations in this study. This finding was in agreement with previous studies [12, 21] which revealed that higher concentrations of CuO NPs exhibited inhibitory effects on the activity of activated sludge microorganisms. In addition, a decrease in the live/dead ratio was observed after 5 h exposure to CuO NPs (2.14) and copper ions (2.08) at high concentration of 50 mg/L, although it was not significantly ($p < 0.05$) different compared to the control (2.20).

It has been extensively reported that the toxicity of CuO NPs to activated sludge would be mainly due to the release of soluble Cu^{2+} ions, and the toxicity of Cu^{2+} ions to microorganisms is well documented. [35, 36] However, our work demonstrated that biosorption of CuO NP onto sludge played a major role in inhibiting bacterial activity and not copper ions dissolution in the bulk. In the present study, only 2.69 mg/L Cu^{2+} was released from CuO NPs which is unlikely to have caused severe inhibition. A release of 1.85 mg/L was observed by Hou et al. [12] when sludge flocs were exposed to CuO NPs at the same initial concentration (50 mg/L). This discrepancy might have been attributed to the size difference of investigated CuO NPs (40 nm ± 5 nm in the present study versus 92±12 nm in Hou et al. [12]), which in turn may lead to the different interaction between NPs and bacteria, as well as the toxicity induced by NPs. Previous studies have reported that CuO NPs could enhance the production of extracellular polymeric substances (EPS), [12] which could strongly interact with the polymer matrix to impede the access of pollutants to the bacterial cells and further increase the toxic resistance of the activated sludge by retarding the contact of the metal with the bacteria within bioflocks. [37] However, once the amount of released metal ions increased, the protective capacity of EPS to impede the access of the CuO NPs to the activated sludge was weakened, due to their loose structure under high toxicity condition. This explains the increased inhibition of CuO NPs to activated sludge at higher concentrations observed in the present study. The toxicity of CuO NPs exposed to bacteria can also be attributed to the changes of the sludge properties. [21] At low concentrations of NPs, the dissolved Cu^{2+} ions from CuO NPs could function as the bridges between the functional groups on the surface of bacteria and help to aggregate the microbes and promote the bio flocculation formation. However, under higher concentrations of CuO NPs, the increased cell surface charge weakened the strength between EPS and cations, resulting in the deterioration of the flocculating ability of activated sludge. Moreover, it has been proven that the toxicity of CuO

NPs could damage the cell membrane of bacteria (e.g., *Escherichia coli)*, which would directly lead to the death of cell. [35, 38]

Conclusions

In this study, the fate and behaviour of CuO NPs and copper ions in the waste activated sludge process were investigated in SBR. The data indicate that the activated sludge process has the potential to remove CuO NPs from wastewater. CuO NPs were efficiently retained by activated sludge and CuO NPs were removed more effectively from the wastewater compared to copper ions. Additionally, CuO NPs exhibited greater biosorption capacity and stronger affinity to sewage sludge than copper salt. The short-term exposure to CuO NPs at 1 mg/L could cause some effects on COD and ammonia removal. The exposure to CuO NPs and Cu^{2+} ions at higher concentrations of 10 mg/L and 50 mg/L caused significant inhibition in biological wastewater treatment. The results of bacterial integrity analysis imply that CuO NPs and copper salt at higher concentrations reduced the viability of bacteria in the biological treatment process.

Acknowledgments

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

References

[1] Kusic, H.; D. Leszczynska; N. Koprivanac; I. Peternel. Role of quantum dots nanoparticles in the chemical treatment of colored wastewater: Catalysts or additional pollutants. J. Environ. Sci., **2011**, *23*(9), 1479-1485.

[2] Dasari, T.P.; K. Pathakoti; H.M. Hwang. Determination of the mechanism of photoinduced toxicity of selected metal oxide nanoparticles (ZnO, CuO, Co3O4 and TiO2) to E. coli bacteria. J. Environ. Sci., **2013**, *25*(5), 882-888.

[3] Gottschalk, F.; T. Sonderer; R.W. Scholz; B. Nowack. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. Environ. Sci. and Technol., **2009**, *43*(24), 9216-9222.

[4] Cioffi, N.; N. Ditaranto; L. Torsi; R.A. Picca; L. Sabbatini; A. Valentini; L. Novello; G. Tantillo; T. Bleve-Zacheo; P.G. Zambonin. Analytical characterization of bioactive fluoropolymer ultra-thin coatings modified by copper nanoparticles. Analytical and Bioanalytical Chemistry, **2005**, *381*(3), 607-616.

[5] Chen, P.C.; G. Shen; C. Zhou. Chemical sensors and electronic noses based on 1-D metal oxide nanostructures. IEEE Transactions on Nanotechnology, **2008**, *7*(6), 668-682.

[6] Ren, G.; D. Hu; E.W.C. Cheng; M.A. Vargas-Reus; P. Reip; R.P. Allaker. Characterisation of copper oxide nanoparticles for antimicrobial applications. Int. J. Antimicrob. Agents, **2009**, *33*(6), 587-590.

[7] Huang, H.L.; H.P. Wang. Speciation of nano-copper collected in molecular sieves from chemical-mechanical planarization wastewater. J. Electron Spectros. and Related Phenomena, **2005**, *144-147*, 307-309.

[8] Brar, S.K.; M. Verma; R.D. Tyagi; R.Y. Surampalli. Engineered nanoparticles in wastewater and wastewater sludge - Evidence and impacts. Waste Manag., **2010**, *30*(3), 504-520.

[9] Boxall, A.B.A.; K. Tiede; Q. Chaudhry. Engineered nanomaterials in soils and water: How do they behave and could they pose a risk to human health? Nanomedicine, **2007**, *2*(6), 919-927.

[10] Ganesh, R.; J. Smeraldi; T. Hosseini; L. Khatib; B.H. Olson; D. Rosso. Evaluation of nanocopper removal and toxicity in municipal wastewaters. Environ. Sci. and Technol., **2010**, *44*(20), 7808-7813.

[11] Limbach, L.K.; R. Bereiter; E. Müller; R. Krebs; R. Gälli; W.J. Stark. Removal of oxide nanoparticles in a model wastewater treatment plant: Influence of agglomeration and surfactants on clearing efficiency. Environ. Sci. and Technol., **2008**, *42*(15), 5828-5833.

[12] Hou, J.; L. Miao; C. Wang; P. Wang; Y. Ao; B. Lv. Effect of CuO nanoparticles on the production and composition of extracellular polymeric substances and physicochemical stability of activated sludge flocs. Biores. Technol., **2015**, *176*, 65-70.

[13] Otero-González, L.; J.A. Field; R. Sierra-Alvarez. Inhibition of anaerobic wastewater treatment after long-term exposure to low levels of CuO nanoparticles. Wat. Res., **2014**, *58*, 160-168.

[14] Luna-delRisco, M.; K. Orupõld; H.C. Dubourguier. Particle-size effect of CuO and ZnO on biogas and methane production during anaerobic digestion. J. Hazard. Mat., **2011**, *189*(1-2), 603-608.

[15] Mortimer, M.; K. Kasemets; A. Kahru. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila.* Toxicology, **2010**, *269*(2-3), 182-189.

[16] Heinlaan, M.; A. Ivask; I. Blinova; H.C. Dubourguier; A. Kahru. Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus.* Chemos., **2008**, *71*(7), 1308-1316.

443 [17] Aruoja, V.; H.C. Dubourguier; K. Kasemets; A. Kahru. Toxicity of nanoparticles of CuO,
444 ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata.* Sci. Total Environ. , **2009**, *407*,
445 1461-1468.
446 [18] Hou, L.; K. Li; Y. Ding; Y. Li; J. Chen; X. Wu; X. Li. Removal of silver nanoparticles in
447 simulated wastewater treatment processes and its impact on COD and NH_4 reduction. Chemos.,
448 **2012**, *87*(3), 248-252.
449 [19] APHA, In *Standard Methods for the Examination of Water and Wastewater 22th edition*.
450 Washington, D.C: 2012.
451 [20] Kaegi, R.; A. Voegelin; C. Ort; B. Sinnet; B. Thalmann; J. Krismer; H. Hagendorfer; M.
452 Elumelu; E. Mueller. Fate and transformation of silver nanoparticles in urban wastewater
453 systems. Wat. Res., **2013**, *47*(12), 3866-3877.
454 [21] Chen, H.; X. Zheng; Y. Chen; M. Li; K. Liu; X. Li. Influence of copper nanoparticles on
455 the physical-chemical properties of activated sludge. PLoS ONE, **2014**, *9*(3).
456 [22] Chaúque, E.F.C.; J.N. Zvimba; J.C. Ngila; N. Musee. Stability studies of commercial
457 ZnO engineered nanoparticles in domestic wastewater. Physics and Chemistry of the Earth, Parts
458 A/B/C, **2014**, *67–69*, 140-144.
459 [23] Hsu, H.F.; M. Kumar; Y.S. Ma; J.G. Lin. Extent of precipitation and sorption during
460 copper removal from synthetic wastewater in the presence of sulfate-reducing bacteria. Environ.
461 Eng. Sci., **2009**, *26*(6), 1087-1096.
462 [24] Vreysen, S.; A. Maes; H. Wullaert. Removal of organotin compounds, Cu and Zn from
463 shipyard wastewaters by adsorption - flocculation: A technical and economical analysis. Marine
464 Poll. Bull., **2008**, *56*(1), 106-115.
465 [25] Dobrevsky, I.; M. Dimova-Todorova; T. Panayotova. Electroplating rinse waste water
466 treatment by ion exchange. Desalination, **1997**, *108*(1-3), 277-280.

[26] Bondarenko, O.; K. Juganson; A. Ivask; K. Kasemets; M. Mortimer; A. Kahru. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: A critical review. Archives of Toxicology, **2013**, *87*(7), 1181-1200.

[27] Karri, S.; R. Sierra-Alvarez; J.A. Field. Toxicity of copper to acetoclastic and hydrogenotrophic activities of methanogens and sulfate reducers in anaerobic sludge. Chemos., **2006**, *62*(1), 121-127.

[28] Chen, Z.; Y. Wang; K. Li; H. Zhou. Effects of increasing organic loading rate on performance and microbial community shift of an up-flow anaerobic sludge blanket reactor treating diluted pharmaceutical wastewater. J. Biosci. and Bioeng., **2014**.

[29] Tan, M.; G. Qiu; Y.P. Ting. Effects of ZnO nanoparticles on wastewater treatment and their removal behavior in a membrane bioreactor. Biores. Technol., **2015**, *185*, 125-133.

[30] Fan, T.; Y. Liu; B. Feng; G. Zeng; C. Yang; M. Zhou; H. Zhou; Z. Tan; X. Wang. Biosorption of cadmium(II), zinc(II) and lead(II) by *Penicillium simplicissimum*: Isotherms, kinetics and thermodynamics. J. Hazard. Mat., **2008**, *160*(2-3), 655-661.

[31] Göksungur, Y.; S. Üren; U. Güvenç. Biosorption of cadmium and lead ions by ethanol treated waste baker's yeast biomass. Biores. Technol., **2005**, *96*(1), 103-109.

[32] Sheng, G.P.; J. Xu; H.W. Luo; W.W. Li; W.H. Li; H.Q. Yu; Z. Xie; S.Q. Wei; F.C. Hu. Thermodynamic analysis on the binding of heavy metals onto extracellular polymeric substances (EPS) of activated sludge. Wat. Res., **2013**, *47*(2), 607-614.

[33] Westerhoff, P.K.; A. Kiser; K. Hristovski. Nanomaterial removal and transformation during biological wastewater treatment. Environ. Eng. Sci., **2013**, *30*(3), 109-117.

[34] Kaegi, R.; A. Voegelin; B. Sinnet; S. Zuleeg; H. Hagendorfer; M. Burkhardt; H. Siegrist. Behavior of Metallic Silver Nanoparticles in a Pilot Wastewater Treatment Plant. Environ. Sci. & Technol., **2011**, *45*(9), 3902-3908.

[35] Zhao, J.; Z. Wang; Y. Dai; B. Xing. Mitigation of CuO nanoparticle-induced bacterial membrane damage by dissolved organic matter. Wat. Res., **2013**, *47*(12), 4169-4178.

[36] Gunawan, C.; W.Y. Teoh; C.P. Marquis; R. Amal. Cytotoxic origin of copper(II) oxide nanoparticles: Comparative studies with micron-sized particles, leachate, and metal salts. ACS Nano, **2011**, *5*(9), 7214-7225.

[37] Ma, J.; X. Quan; X. Si; Y. Wu. Responses of anaerobic granule and flocculent sludge to ceria nanoparticles and toxic mechanisms. Biores. Technol., **2013**, *149*, 346-352.

[38] Duan, J.; Y. Yu; Y. Li; Y. Yu; Y. Li; X. Zhou; P. Huang; Z. Sun. Toxic Effect of Silica Nanoparticles on Endothelial Cells through DNA Damage Response via Chk1-Dependent G2/M Checkpoint. PLoS ONE, **2013**, *8*(4).

FIGURE CAPTIONS

Figure 1. CuO NPs (A-C) in deionized water at different resolution (i.e., 500, 100 and 50 nm) characterized by TEM. These are representive images of particles after drying the suspension on the microscope grid which resulted in aggregation.

Figure 2. Kinetics of Cu^{2+} released from CuO NPs (A) and Cu^{2+} released from $CuSO_4$ (B). Error bars represent standard deviations of triplicate measurements.

Figure 3. COD concentrations in the effluent of A) CuO NPs treatment; and B) $CuSO_4$ treatment. Error bars represent standard deviations of triplicate measurements.

Figure 4. NH_4-N concentrations in the effluent of A) CuO NP treatment; and B) $CuSO_4$ treatment. Error bars represent standard deviations of triplicate measurements.

Figure 5. Cu^{2+} concentrations in the biosolids for A) CuO treatment; and B) $CuSO_4$ treatment. Error bars represent standard deviations of triplicate measurements.

Figure 6. SEM images of activated sludge after CuO NPs and Cu^{2+} ions exposure at the concentration of 10 mg/L after 5 h. A) Sludge in the control; B) Sludge in the treatment exposed to CuO NPs; and C) Sludge in the treatment exposed to Cu^{2+} ions; D) EDS spectra for A); E) EDS spectra for B); and F) EDS spectra for C).

Figure 7. Bacterial viability in A) control treatment; B) in activated sludge exposed to CuO NPs at the concentration of 50 mg L^{-1}; and C) in activated sludge exposed to $CuSO_4$ treatment at the concentration of 100 mg L^{-1} at the end of the experiment using confocal microscopy.

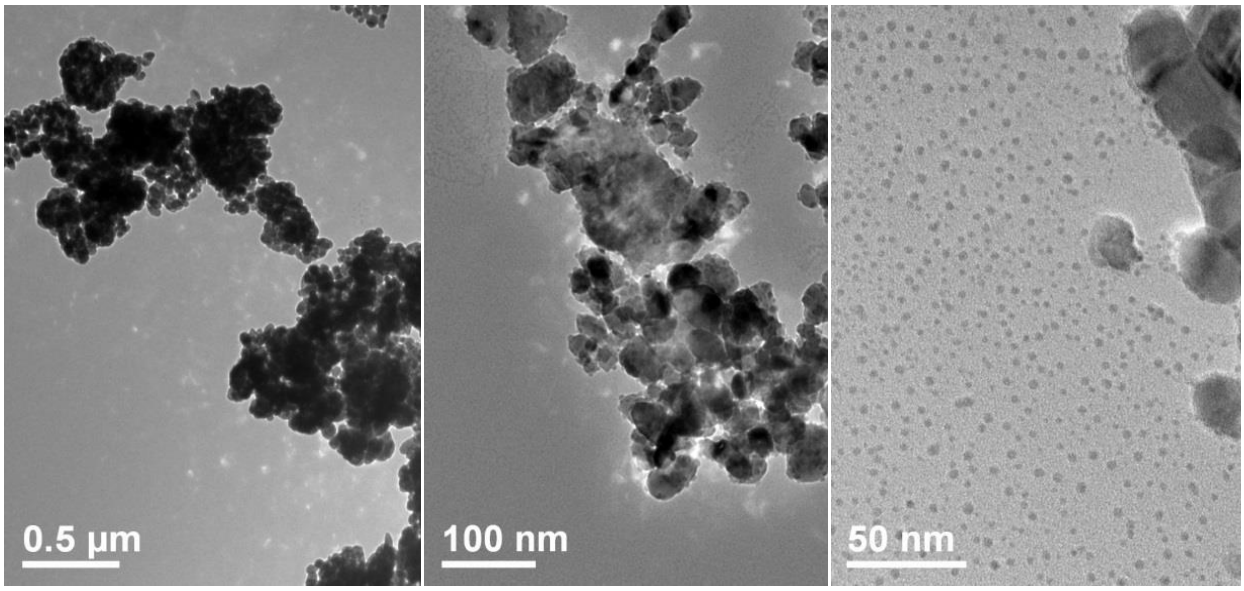
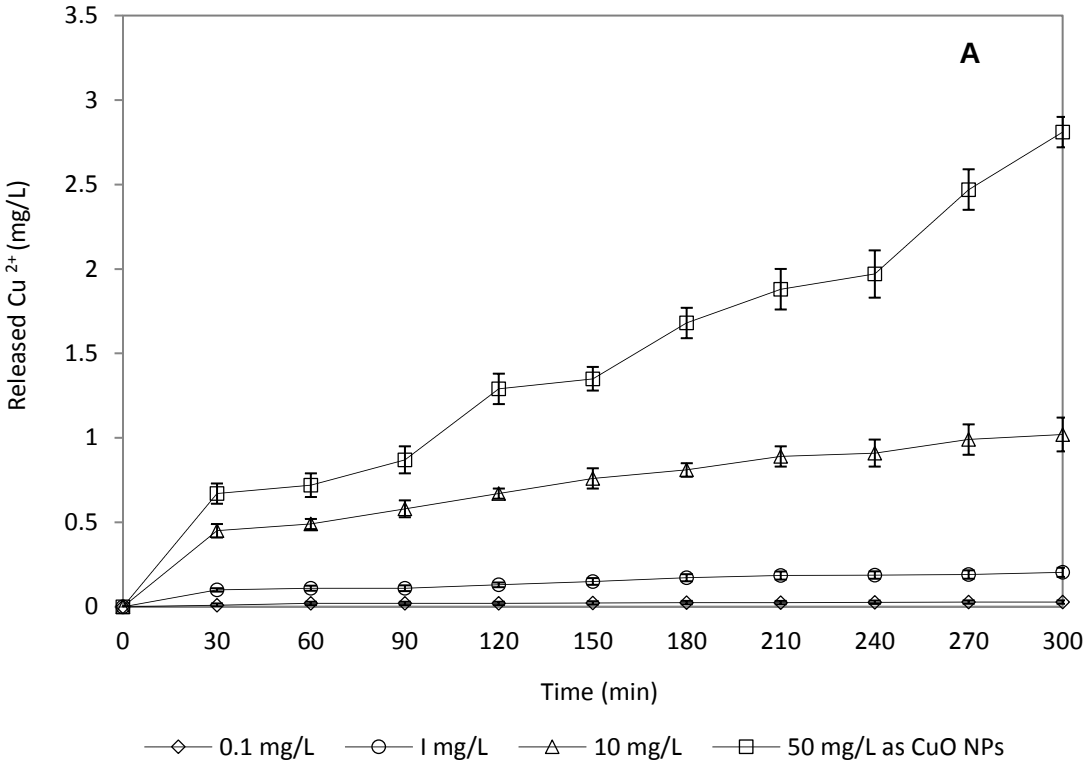
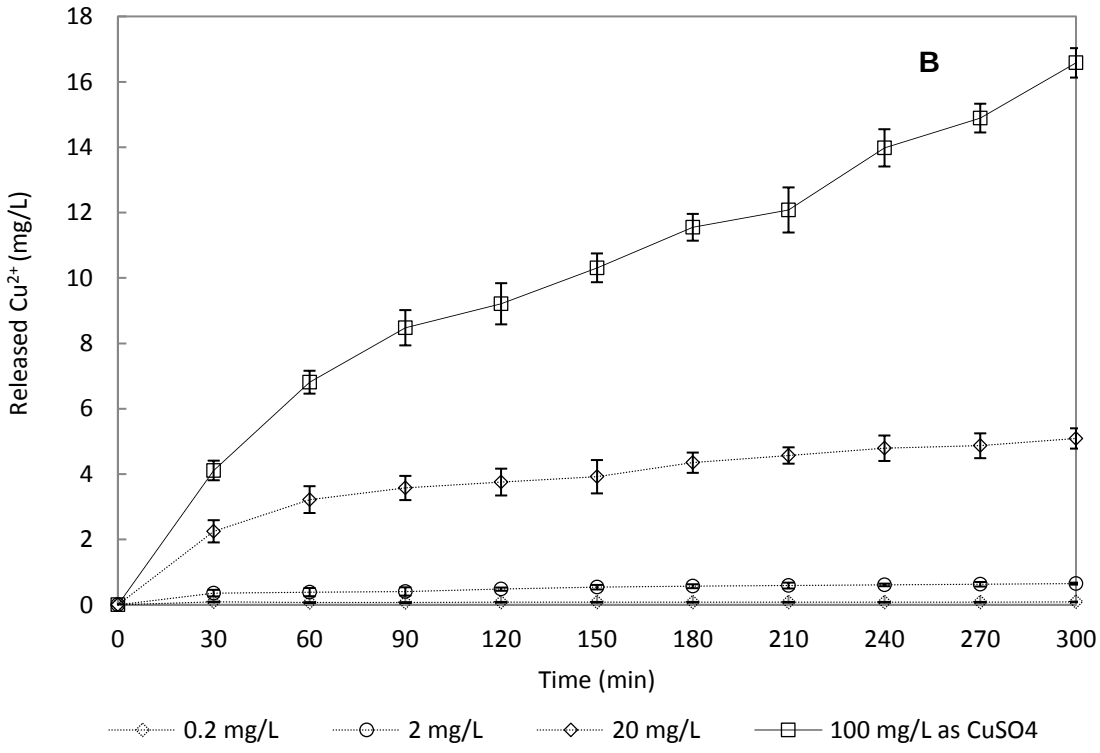


Fig. 1



534

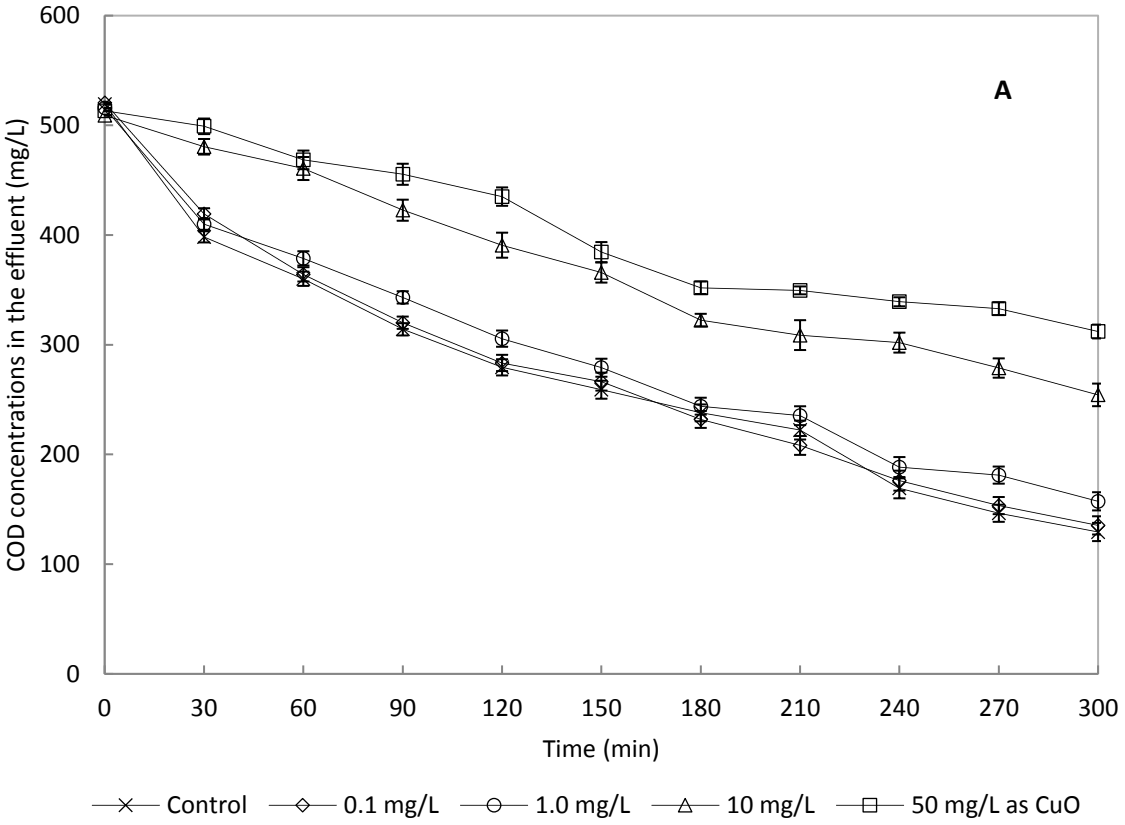
535



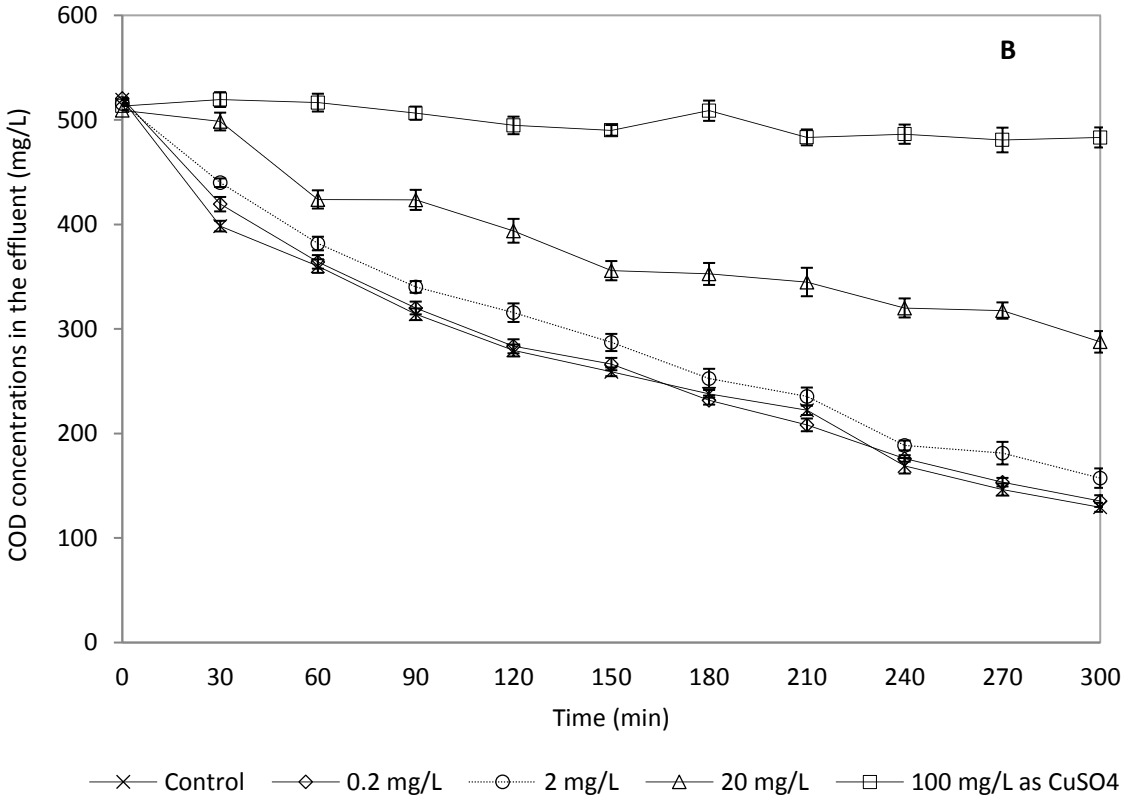
536

537 Fig. 2

538



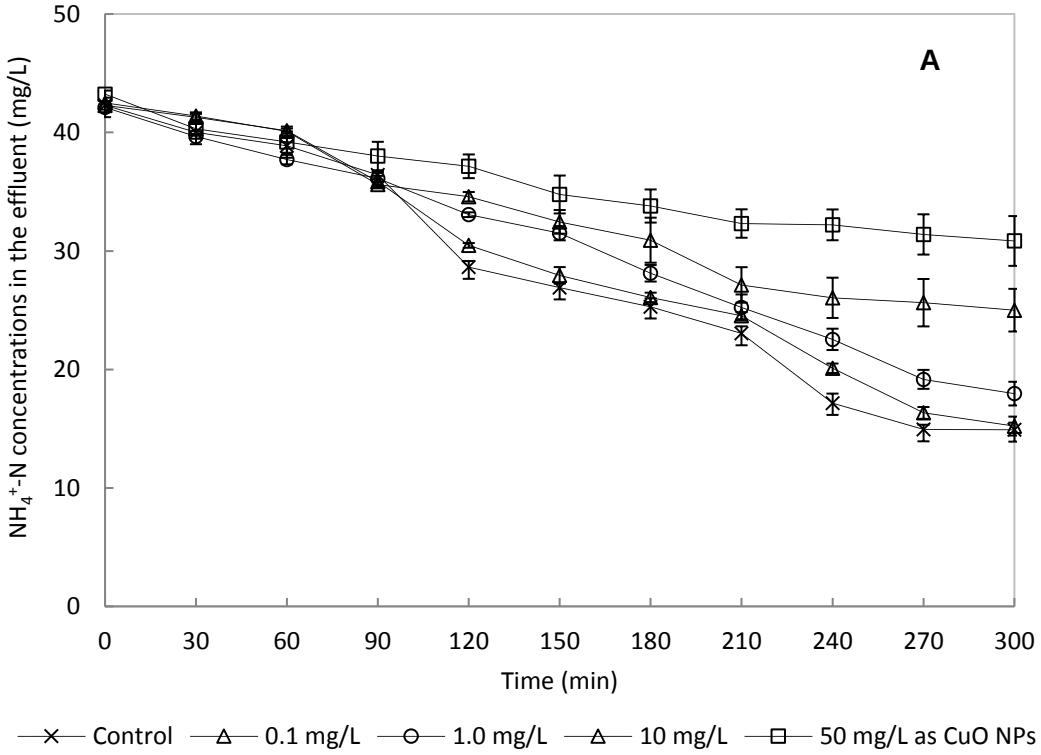
539



540

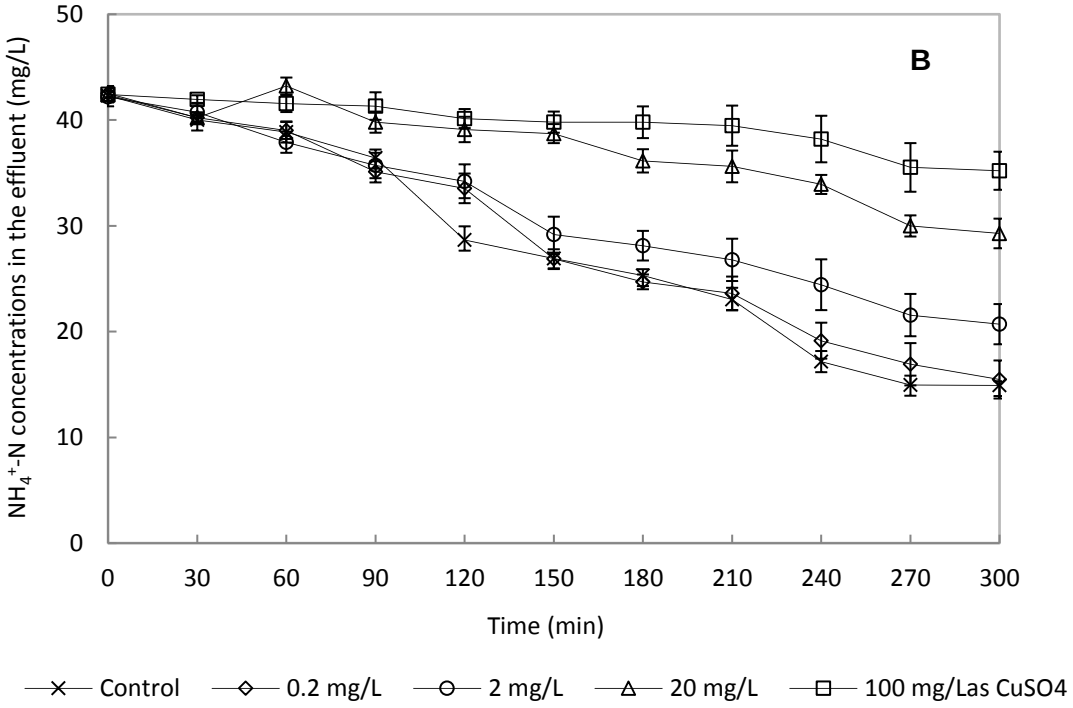
541 Fig. 3

542



543

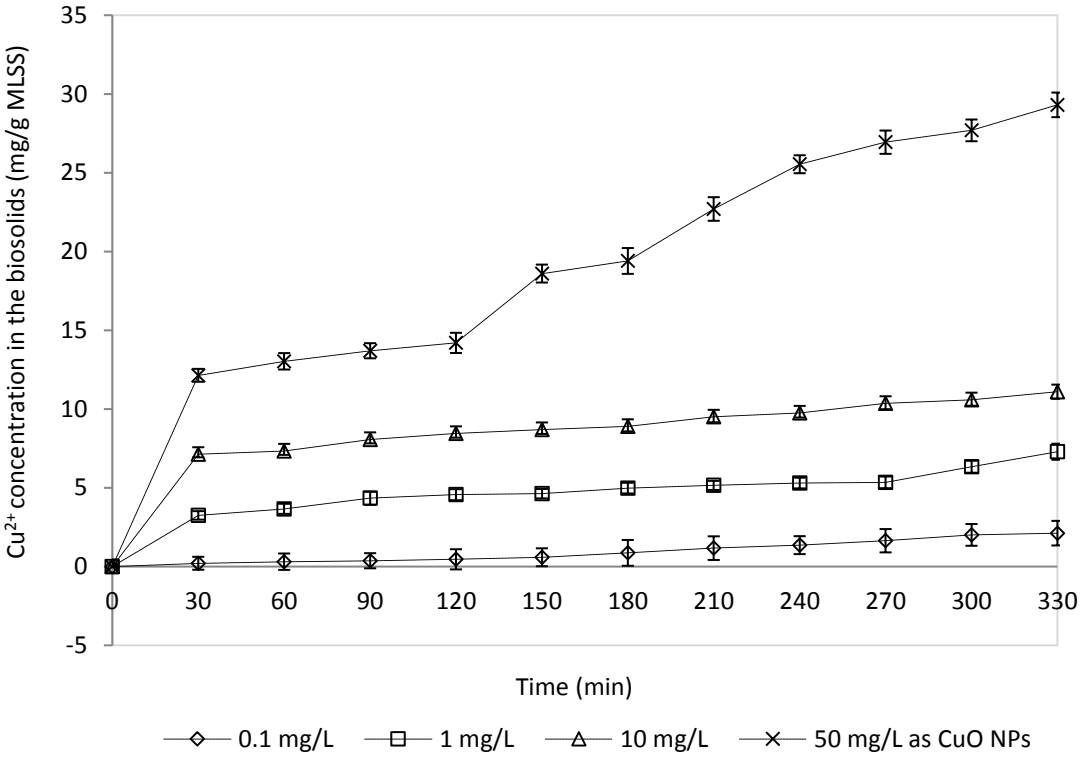
544



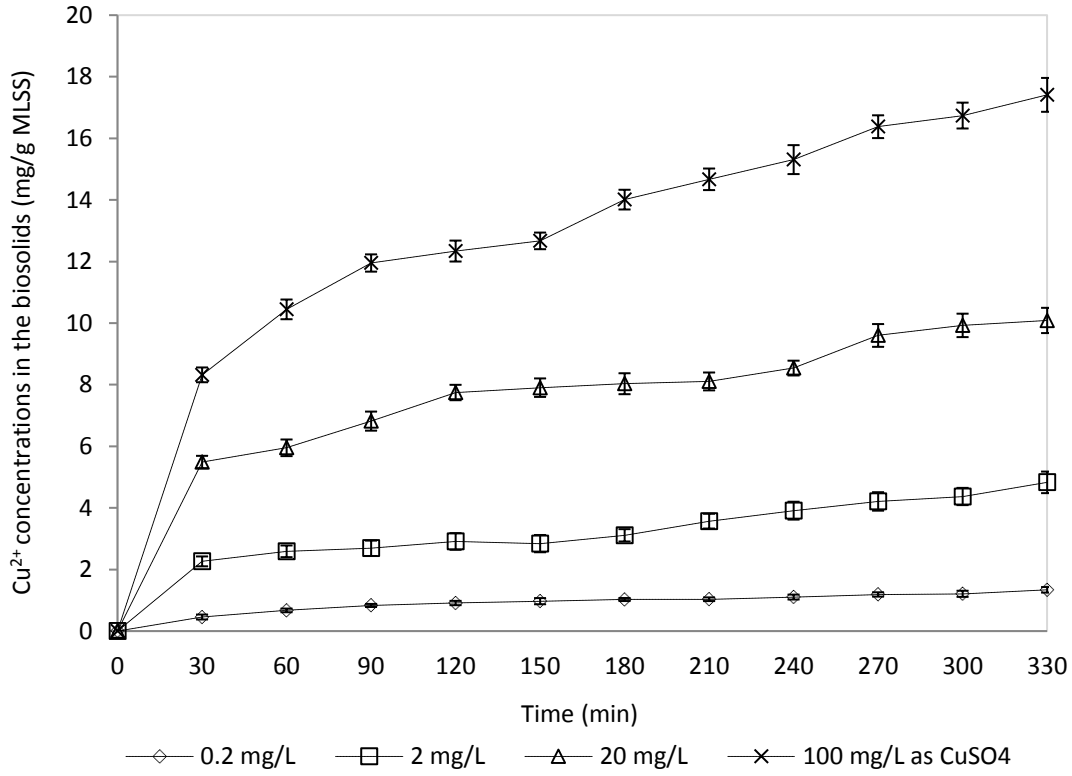
545

546 Fig. 4

547



548



549

550 Fig. 5

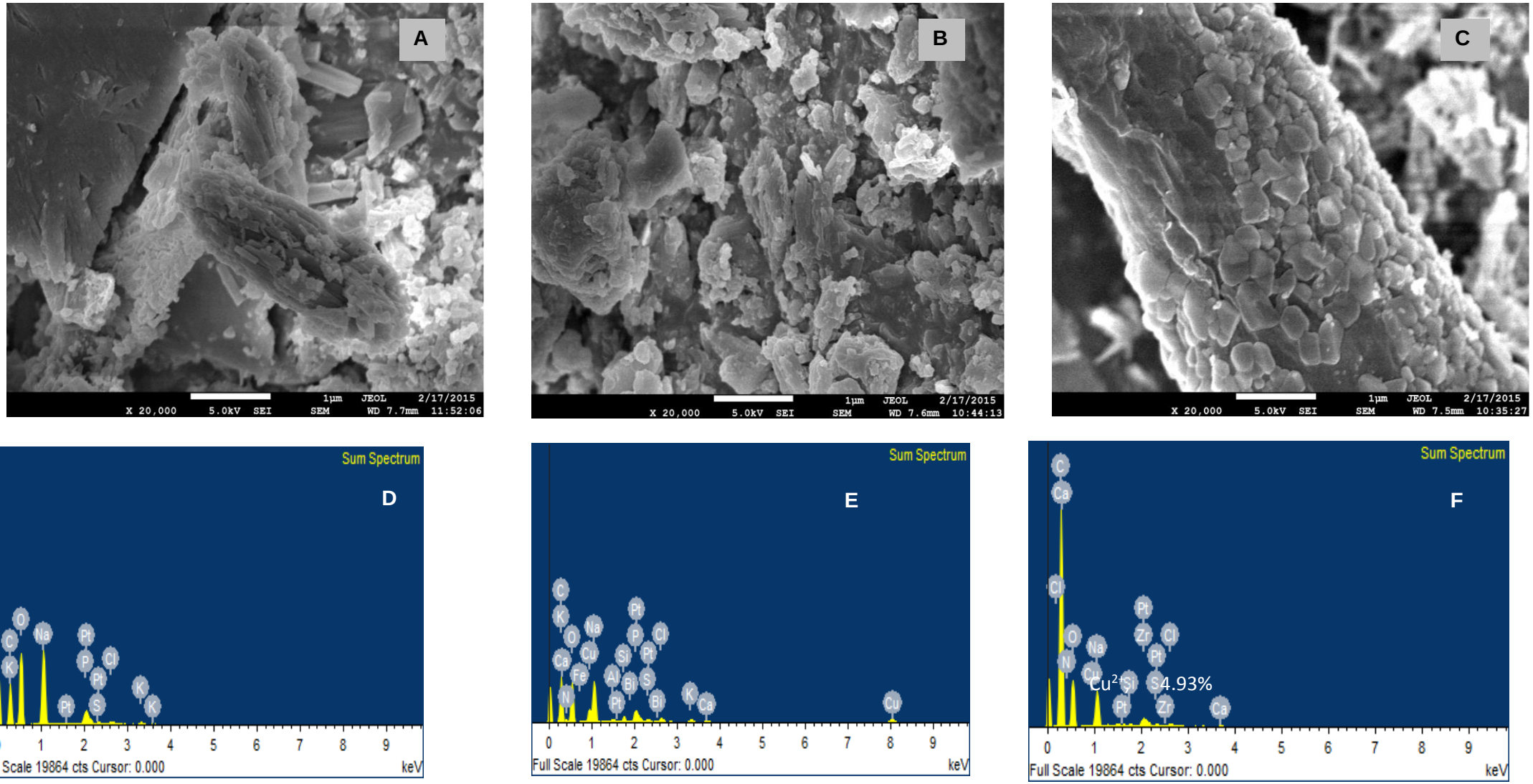
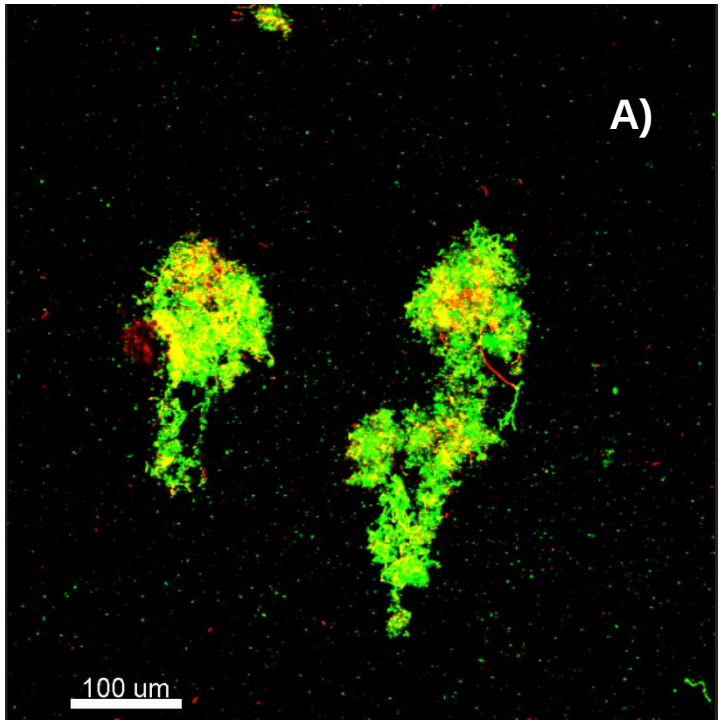
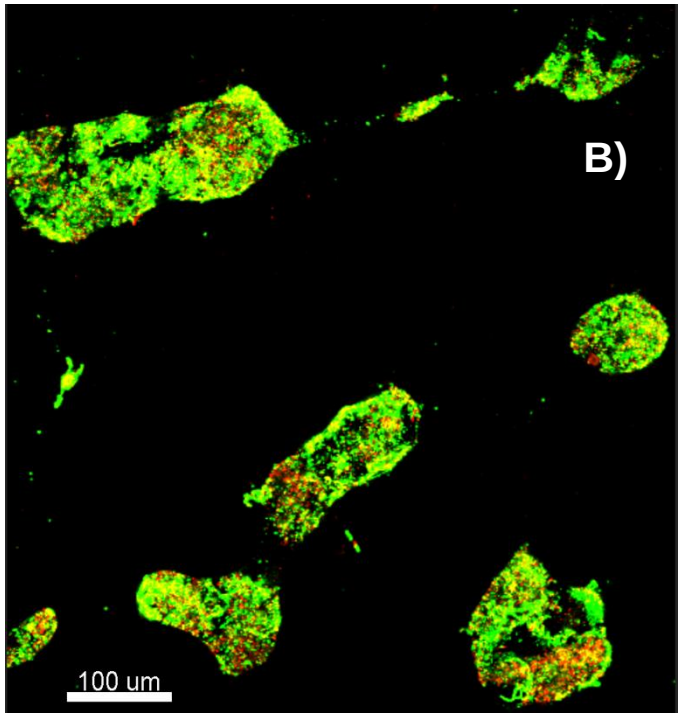
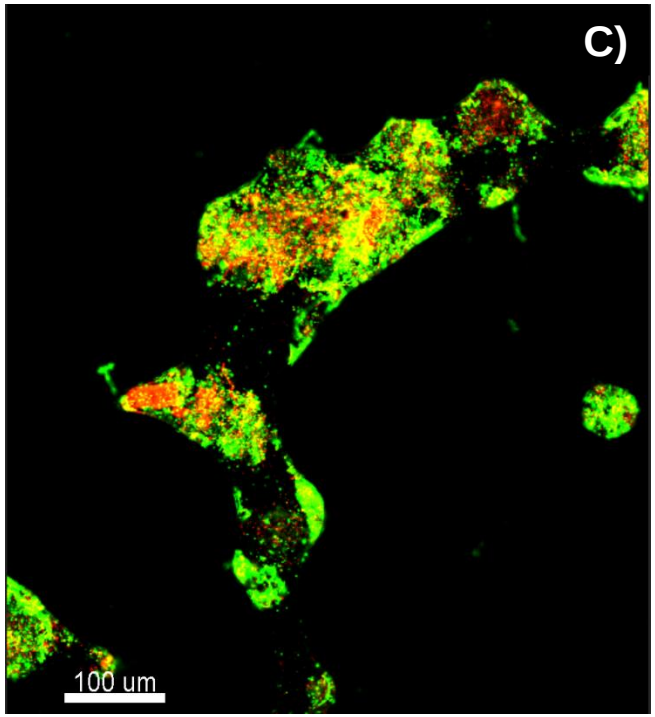

A
X 20,000 5.0kV SEI SEM WD 7.7mm 11:52:06
1µm JEOL 2/17/2015
B
X 20,000 5.0kV SEI SEM WD 7.6mm 10:44:13
1µm JEOL 2/17/2015
C
X 20,000 5.0kV SEI SEM WD 7.5mm 10:35:27
1µm JEOL 2/17/2015
Sum Spectrum
D
O C K Na Pt P Cl Pt Pt S K K
0 1 2 3 4 5 6 7 8 9
Full Scale 19864 cts Cursor: 0.000
keV
Sum Spectrum
E
C K O Na Ca Cu Fe N Si Al Pt P Pt Cl Pt Bi S Pt Bi K Ca Cu
0 1 2 3 4 5 6 7 8 9
Full Scale 19864 cts Cursor: 0.000
keV
Sum Spectrum
F
C Ca Cl O N Na Cu Cu2+ Si S 4.93% Pt Pt Zr Cl Pt Zr Ca
0 1 2 3 4 5 6 7 8 9
Full Scale 19864 cts Cursor: 0.000
keV

Fig. 6


A)
100 um


B)
100 um



551

552 Fig. 7

553