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3 **Comparison and Distribution of Copper Oxide Nanoparticles and Copper Ions in**
4 **Activated Sludge Reactors.**
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26 **Abstract**

27

28 Copper oxide nanoparticles (CuO NPs) are increasingly applied in the industry which results
29 inevitably in their release of these materials into the hydrosphere. In this study, simulated waste
30 activated sludge experiments were conducted to investigate the effects of Copper Oxide NPs at
31 concentrations of 0.1, 1, 10 and 50 mg/L and compare it with its ionic counterpart (as CuSO₄). It
32 was found that 0.1 mg/L CuO NPs had negligible effects on Chemical Oxygen Demand (COD)
33 and ammonia removal. However, the presence of 1, 10 and 50 mg/L CuO NPs decreased COD
34 removal from 78.7% to 77%, 52.1% and 39.2%, respectively ($p < 0.05$). The corresponding
35 effluent ammonium (NH₄-N) concentration increased from 14.9 mg/L to 18, 25.1 and 30.8 mg/L,
36 respectively. Under equal Cu concentration, copper ions were more toxic towards
37 microorganisms compared to CuO NPs. CuO NPs were removed effectively (72-93.2%) from
38 wastewater due to a greater biosorption capacity onto activated sludge, compared to the copper
39 ions (55.1%-83.4%). The SEM images clearly showed the accumulation and adsorption of CuO
40 NPs onto activated sludge. The decrease in Live/dead ratio after 5 h exposure of CuO NPs and
41 Cu²⁺ indicated the loss of cell viability in sludge flocs.

42

43 **Keywords:** CuO nanoparticles; copper ions; waste activated sludge; biosorption

44

45 **Introduction**

46

47 Nanotechnology has become very popular over the last few decades due to significant advances
48 with applications in medicine and semiconductor, chemical and electronics industries. ^[1-3] As one
49 of the most important engineered applications, copper oxide nanoparticles (CuO NPs) exhibit
50 optical, electrical and catalytic properties, and have been used intensively in electronics,

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

57 Results from material flow analyses suggest that a major fraction of the NPs in commercial
58 products will eventually enter municipal or industrial wastewaters, and subsequently reach
59 wastewater treatment plants (WWTPs). ^[8, 9] WWTPs are considered as the last barriers prior to
60 their environmental release. ^[10] Therefore, efficient removal of engineered NPs from wastewater
61 is particularly important in view of their increasing evidence for their ecotoxicity. ^[11]

62 Furthermore, their toxicity to some microorganisms within the biological systems of WWTPs is
63 of particular concern, since the inhibition and loss of certain bacterial species involved could be
64 detrimental to biological treatment performance. ^[12] Previous study by Otero-González et al. ^[13]
65 indicated that the extended exposure to even relatively low concentration (1.4 mg/L) of CuO NPs
66 had a markedly negative effect on the performance of methanogenesis in upflow anaerobic
67 sludge blanket (UASB) reactor. In another recently study, 50% inhibition of CH₄ production was
68 also observed during anaerobic digestion processes in the presence of 11 mg Cu L⁻¹ of CuO NPs
69 over a 14-d period. ^[14]

70 In addition, the fate, transport, and toxicity of NPs in wastewater treatment processes may differ
71 largely from those of their ionic counterparts, due to the differences in the properties (size,
72 charge density), chemical composition of media (pH, organics, ionic strength), test conditions,
73 and organisms evaluated. ^[10] CuO NPs and Cu²⁺ ions were reported to show different toxicity to
74 some microbes. ^[15, 16] In a recent study of the toxic effects of CuO NPs, bulk CuO and CuSO₄ on
75 *Tetrahymena thermophila*, Mortimer et al. ^[15] indicated that the most toxic Cu compound was

76 CuSO₄, which was approximately 120 times more toxic than CuO NPs and 1500 times more
77 toxic than bulk CuO. The different toxicity of Cu compounds has also been reported in a study of
78 Heinlaan et al. ^[16] where the EC₅₀ values for bulk CuO, CuO NPs and CuSO₄ were 3811, 79, 1.6
79 mg/L (*Vibrio fischer*); 165, 3.2, 0.17 mg/L (*Daphnia magna*); and 95, 2.1, 0.11 mg/L
80 (*Thamncephalus platyurus*), respectively. However, Aruoja et al. ^[17] investigated the toxicities
81 of ZnO, TiO₂ and CuO NPs to microalgae *Pseudokirchneriella subcapitata* and reported that the
82 bioavailable EC₅₀ values of CuO NPs were not significantly different from the EC₅₀ of CuSO₄
83 (0.02 mg Cu/L).

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

93 Therefore, the objectives of this study were (a) to compare the short term effects and fate of CuO
94 NPs and Cu²⁺ in a laboratory scale waste activated sludge process fed with real wastewater; (b)
95 to investigate the effects of 0.1, 1, 10 and 50 mg/L CuO NPs on COD and nitrogen removals; (c)
96 to determine the accumulation of Cu ions in the effluent and onto activated sludge over short
97 term experiments; (d) to determine the morphology of activated sludge using Scanning electron
98 microscopy (SEM); (e) to assess the impacts of the presence of CuO NPs and Cu²⁺ ions on
99 bacterial integrity using the Live/Dead *Ba*clight bacterial viability technique which was not used

100 previously in particular under short term experiments (5 hours) at concentrations as high at 50
101 mg/L.

102

103 **Materials and methods**

104

105 *Activated sludge and wastewater*

106

107 Primary wastewater was collected from Ulu Pandan Water Reclamation Plant (WRP), Singapore.
108 The total treatment capacity of Ulu Pandan WRP is 361,000 m³ per day. The treatment process
109 includes typical preliminary, primary and secondary treatment processes. The wastewater was
110 collected from the effluent of the primary sedimentation tank. As Ulu Pandan WRP treats
111 combined industrial and domestic wastewater, the contaminant concentrations are expected to be
112 higher than those in common domestic WWTPs. Real wastewater was stored at 4°C until it was
113 fed to the SBRs.

114

115 *CuO NPs characterization*

116

117 The CuO NPs were purchased from Sigma-Aldrich (Singapore) with average particles size of
118 40±5 nm. CuO NPs stock solutions (100 mg/L) were prepared by adding dry particles into Milli-
119 Q (pH=6.8±0.2), and then the suspensions were sonicated (30°C, 100 W, 40 kHz) for 30 min and
120 shaken for 2 h to increase their dispersion. *Zeta* potential of CuO NPs in the suspensions were
121 measured using a Nanosizer (Malvern Instruments Ltd., UK). The morphology of the CuO NPs
122 was examined using transmission electron microscopy (TEM) (JEOL JEM-3010, Japan). To
123 avoid agglomeration or aggregation, water bath ultrasonic treatment was carried out to increase
124 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 CuSO₄) at concentration of 0.2, 2.0, 20, and 100 mg/L CuSO₄/L such
143 that both sets of SBR contained exactly 0.08, 0.8, 8.0 and 40.0 mg Cu²⁺/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

150 Sampling commenced after 15 days of operation of reactor, in order to ensure stable operation.
151 Aliquots of completely mixed liquor suspensions were collected every 0.5 h over a period of 5 h.
152 Collected samples were first centrifuged for 20 min at 10,000 rpm (Eppendorf 5810R). The
153 measurement of MLSS, mixed liquor volatile suspended solids (MLVSS), chemical oxygen
154 demand (COD), ammonium ($\text{NH}_4^+\text{-N}$), and phosphate (PO_4^{3-}) was in accordance with the
155 Standard Methods. ^[19] All chemical tests were done in triplicate.

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

165

166 ***Bacterial viability assay***

167

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

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

180

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

182

183 Samples were investigated using TEM and SEM. In the first case TEM grids were prepared by
184 placing a drop of suspension (mixed liquor or supernatant) on a holey carbon grid and drawing
185 the suspension through the TEM grid using a paper tissue. The TEM grids were washed
186 afterwards in a drop of distilled water to remove the dissolved compounds. ^[20] The TEM was
187 operated at 200 kV to detect and characterize aggregation state of NPs in the solution.

188 To prepare SEM image, mixed liquor was first washed 3 times with 0.1 M phosphate buffer
189 solution (PBS) (pH 7.7) and fixed in 0.1 M phosphate buffer (7.4) containing 2.5%

190 glutaraldehyde at 4 °C for 4 h. The dried samples were coated with platinum before SEM

191 analysis according to Zheng et al. (2011). The elemental analysis of the particles was carried out
192 using an energy-dispersive X-ray spectroscope (EDS).

193

194 *Statistical analysis*

195

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

200

201 **Results and discussion**

202

203 *Characterization of CuO NPs*

204

205 Figure 1 shows the TEM image of CuO NPs in deionized water under different magnifications
206 (0.5 μm , 100 nm and 50 nm). In the present study, due to their small size and huge surface area,
207 NPs tend to aggregate or agglomerate in aqueous phase. Although the CuO NPs used in this
208 study have a diameter size within the nanometer range, some aggregates of different sizes were
209 formed in the solution where the particles were suspended, even after sonication. The *zeta*
210 potential was -41.7 mV at pH= 6.8 and -35.6 mV at pH=6.4 at the beginning and end of the
211 experiment, respectively.

212

213 *Removal of CuO NPs and copper ions*

214

215 The Cu levels in the biomass-free effluent spiked with CuO NPs and copper salt is shown in
216 Figure 2A. After 5 h exposure, the concentrations of released soluble Cu^{2+} were 0.028, 0.204,
217 1.02 and 2.81 mg/L at the initial CuO NP concentration of 0.1, 1.0, 10 and 50 mg/L, respectively.
218 This finding indicates that the majority of the Cu in the influent was adsorbed onto settled
219 biomass. At the CuO NP concentrations of 0.1 and 1.0 mg/L, both supernatant and effluent Cu
220 content were consistently low. The higher concentrations of released Cu^{2+} observed at the initial
221 CuO NP concentrations of 10 mg/L and 50 mg/L can be attributed to the increased sludge
222 surface charge and the decreased hydrophobicity resulting in more Cu^{2+} ions released from CuO
223 NPs. [21] Furthermore, the Cu concentrations in copper salt treatment were 3.2, 3.1, 4.9 and 5.9
224 fold higher than in the corresponding CuO NPs treatment (Fig. 2B). Less Cu^{2+} was released from

225 NP possibly because humic acids are able to stabilize nanoparticles and retard dissolution rates.

226 [22]

227 Interestingly, CuO NPs were removed more efficiently than copper salt in this study with

228 removal efficiencies ranging from 72% to 93.2% for CuO NPs, while the values were 55.1% to

229 83.4% for Cu²⁺ ions treatment, suggesting that large fraction of CuO NPs was removed from the

230 wastewater. These observations also support the hypothesis that the mechanisms governing the

231 removal of CuO NPs and ionic copper are different. As for copper salt, it is highly possible that

232 the majority of the added copper salt may quickly undergo a transformation due to their

233 dissolution followed by complexation or precipitation. [10, 23] Furthermore, depending on the

234 wastewater characteristics, copper can also be removed by coagulation or ion exchange in

235 wastewaters. [24, 25] In contrast, the attenuation of the CuO NP concentration in the liquid is most

236 likely due to aggregation, settling and biosorption onto the biomass. [12, 26, 27]

237

238 *Effect of CuO NPs and copper ions on COD removal*

239

240 Prior to addition of CuO NPs, the COD concentration in the effluent was around 130 mg/L

241 which corresponds to a COD removal efficiency of 78.7% (Fig. 3). The presence of CuO NPs,

242 however, influenced the COD removal efficiencies, which slightly decreased to 77% ($p < 0.05$) at

243 CuO NP concentrations of 1 mg/L, respectively. The exposure to 10 and 50 mg/L CuO NPs

244 further decreased COD removal efficiencies to 52.1% and 39.2%, respectively. The lower COD

245 removals was due to the high toxicity of the released Cu²⁺ ions from CuO NPs which inhibited

246 microorganisms. It can also be explained by the increased cell surface charge resulting in

247 reduced hydrophobicity and floc breakage as suggested by previous studies. [28, 29] Our finding

248 implies that 1 mg/L CuO NPs will cause some disturbance to the waste activated sludge process

249 which was not reported previously. This finding is in disagreement with Tan et al. [29] who

250 revealed that both short- and long term exposure of 1.0 mg/L of ZnO NPs did not significantly
251 impact COD removal, despite the fact that ZnO NPs may exhibit more toxic effects on specific
252 microorganisms than CuO NPs. Chen et al. [21] investigated the influence of Cu NPs on the
253 physical-chemical properties of activated sludge, and indicated that lower Cu NPs concentrations
254 (5 mg/L) did not affect the sludge properties, while higher Cu NPs concentrations (30-50 mg/L)
255 may deteriorate the physical-chemical properties of activated sludge.

256 When CuSO₄ was used, the Cu⁺² concentration quickly increased to 4.1 mg/L after only 30
257 minutes and gradually increased to 16.6 mg/L after 300 minutes, which resulted in a greater
258 toxicity. In this study, in the presence of 20 and 100 mg/L copper sulphate, COD removals were
259 44.8% and 7.3%, which were significantly ($p < 0.05$) lower than those (52.1% and 39.2%) in the
260 presence of CuO NPs, showing that copper salt exhibited more severe toxicity towards microbes
261 than CuO NPs. Moreover, the MLSS concentration decreased markedly to 1.2 g/L with 100
262 mg/L CuSO₄ (data not shown), showing that flocs were disrupted and cell lysis took place. From
263 Figures 2 and 3, it is clear that CuO NPs is less toxic than CuSO₄ due to the fact that Cu ions
264 from CuSO₄ dissolve more readily in water. These findings are consistent with Heinlaan et al. [16]
265 who evaluated the eco-toxicity of ZnO NPs, CuO NPs and TiO₂ to bacteria and crustaceans, and
266 reported that CuSO₄ was approximately 100-fold more toxic than nano CuO to *Vibrio fischer*
267 with LC₅₀ value of 1.6 versus 79 mg/L, and 1000-fold more toxic than nano CuO to *Daphnia*
268 *magna* (0.17 versus 164.8 mg/L) and *Thamncephalus platyurus* (0.11 versus 94.5 mg/L). In this
269 study, after the addition of 50 mg/L CuO-NPs (equivalent to 40 mg/L Cu⁺²), the measured Zn²⁺
270 concentration in the effluent progressively increased to only 2.8 mg/L after 5 hours, indicating a
271 low dissolution potential of ZnO-NPs in the system, and that the most likely cause of inhibition
272 was therefore the adsorption of CuO NP onto bacterial cells.

273

274 ***Effect of CuO NPs and copper ions on ammonium removal***

275

276 The effect of CuO NPs and copper ions on $\text{NH}_4^+\text{-N}$ removal are shown in Figure 4. The $\text{NH}_4^+\text{-N}$
277 removal in the presence of 0.1 (64.1%) were relatively stable with increasing exposure time and
278 not statistically different ($p < 0.05$) from the negative control at (64.8%) over a period of 5 h
279 exposure. However, when activated sludge was exposed to 1, 10 and 50 mg/L CuO NPs, the
280 effluent $\text{NH}_4^+\text{-N}$ significantly ($p < 0.05$) increased from 14.9 mg/L (control) to 18 mg/L, 25.1
281 mg/L and 30.8 mg/L, respectively, suggesting that CuO NPs at 1 mg/L could start causing some
282 inhibition to ammonia oxidizing bacteria. At higher CuO NP concentration, the flocculating
283 ability deteriorated due to the increased cell surface charge and the decreased hydrophobicity
284 made the sludge flocs more dispersed, which further increased the toxicity of the CuO NPs by
285 increasing the contact between CuO NPs and bacteria. [21] This finding also indicated that
286 biosorption of CuO NPs onto activated sludge induced adverse effects on the diversity and
287 activity of nitrifying microbial species. Additionally, in the present study, effluent ammonia
288 concentration (20.7 mg/L, 29.3 mg/L and 35.2 mg/L, respectively) in the presence of CuSO_4
289 were higher than those in the presence of ZnO NPs (18 mg/L, 25.1 mg/L and 30.8 mg/L,
290 respectively), implying that Cu^{2+} ions exhibited more severe toxicity to ammonia oxidizing
291 bacteria than ZnO NPs.

292

293 *Accumulation of CuO NPs and copper ions onto activated sludge*

294

295 Activated sludge biomass from biological wastewater treatment processes is able to remove
296 heavy metals from wastewater, and biosorption plays an important role in heavy metal recovery.
297 [30, 31] CuO NPs and dissolved Cu^{2+} have been observed to bind on the surface of activated sludge.
298 [32] Previous studies reported that biosorption of CuO NPs can take place in activated sludge
299 treatment [12] and anaerobic sludge treatment exposed to synthetic wastewater. [13] Different

300 mechanisms of partitioning of NPs to biosolids have been identified including binding to
301 extracellular polymers or cell surface, active cellular uptake, entrapment into flocs and diffusion
302 into biofilms. [33] In the present study, a gradual increase in the Cu^{2+} concentrations in the
303 biosolids was observed for both CuO NPs and copper salt treatment (Fig. 5). The copper
304 concentrations were 2.12, 7.29, 11.1 and 29.31 mg/g MLSS at the CuO NP concentrations of 0.1,
305 1.0, 10 and 50 mg/L after 5 h exposure, respectively, which was 1.58, 1.51, 1.10 and 1.68 fold
306 more than in the CuSO_4 treatment. At 50 mg/L exposure, a mass balance on Zn revealed that 98%
307 of Cu from CuO NPs ended up in biosolids and 2% in the effluent. For CuSO_4 , the mass balance
308 was 86% onto biosolids and 14% in effluent. This finding suggests that CuO NPs have greater
309 potential for adsorption onto biosolids compared to Cu^{2+} ions, due to its smaller particles size
310 and larger surface area, and this biosorption capacity increased with the concentration of CuO
311 NPs. Furthermore, the higher copper levels found in the biosolids were mainly attributed to CuO
312 NPs, instead of the released Cu^{2+} from CuO NPs, given the fact that CuO NPs have much less
313 Cu^{2+} release capacity, compared to copper salt. This finding also reinforces the results of
314 previous studies [11, 34] which indicated that the primary process of NP removal from wastewater
315 is believed to be associated with biosorption onto biomass, although NPs may undergo
316 transformation (e.g., dissolution of metal ions from metal-based NPs). In addition, these
317 observations also support the hypothesis that different mechanisms might govern the removal of
318 CuO NPs and Cu^{2+} ions from wastewater. As for CuO NPs, the attenuation of the CuO NP
319 concentration in the solution phase is most likely due to precipitation of Cu species and CuO NP
320 adsorption onto the biomass. In contrast, copper salt quickly undergo dissolution followed by
321 complexation and precipitation.

322

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

325 the accumulation and adsorption of CuO NPs onto activated sludge. Such observation
326 corroborates previous study assessing the effect of CuO NPs on physicochemical stability of
327 activated sludge flocs. [12] SEM images revealed differences in damage extent between CuO NPs
328 and copper salt. Although these damage extent cannot be accurately quantified based on our
329 SEM analyses, the ionic copper appeared to have transformed to larger size aggregates during
330 the experiment. The accumulation of CuO NPs and Cu²⁺ on activated sludge was also confirmed
331 using EDS profile analysis to confirm their Cu-based composition (Fig. 6D-6E). The EDS profile
332 clearly demonstrates a Cu peak that is absent in the sample from the control reactor.

333

334 ***Bacterial viability assay***

335

336 Figure 7 displays the bacterial viability in the control and in the activated sludge exposed to CuO
337 NPs and copper salt for 5 h. Compared to the control (Fig. 7A), the density of the dead cells
338 increased after the exposure of the activated sludge to 50 mg/L of CuO NPs (Fig. 7B) or 100
339 mg/L Cu²⁺ ions (Fig. 7C), indicating a loss in the cell viability. The structure of the activated
340 sludge became loose with numerous small aggregates of bacterial cells which may result in
341 dispersed flocs. This can be due to the adsorption of NPs onto the sludge and inhibition of cell
342 activity after exposure to 50 mg/L ZnO NPs. This was supported by the significant reduction in
343 contaminant removal observed under the exposure to CuO NPs and copper ions at higher
344 concentrations in this study. This finding was in agreement with previous studies [12, 21] which
345 revealed that higher concentrations of CuO NPs exhibited inhibitory effects on the activity of
346 activated sludge microorganisms. In addition, a decrease in the live/dead ratio was observed after
347 5 h exposure to CuO NPs (2.14) and copper ions (2.08) at high concentration of 50 mg/L,
348 although it was not significantly ($p < 0.05$) different compared to the control (2.20).

349 It has been extensively reported that the toxicity of CuO NPs to activated sludge would be
350 mainly due to the release of soluble Cu²⁺ ions, and the toxicity of Cu²⁺ ions to microorganisms is
351 well documented. [35, 36] However, our work demonstrated that biosorption of CuO NP onto
352 sludge played a major role in inhibiting bacterial activity and not copper ions dissolution in the
353 bulk. In the present study, only 2.69 mg/L Cu²⁺ was released from CuO NPs which is unlikely to
354 have caused severe inhibition. A release of 1.85 mg/L was observed by Hou et al. [12] when
355 sludge flocs were exposed to CuO NPs at the same initial concentration (50 mg/L). This
356 discrepancy might have been attributed to the size difference of investigated CuO NPs (40 nm ±
357 5 nm in the present study versus 92±12 nm in Hou et al. [12]), which in turn may lead to the
358 different interaction between NPs and bacteria, as well as the toxicity induced by NPs. Previous
359 studies have reported that CuO NPs could enhance the production of extracellular polymeric
360 substances (EPS), [12] which could strongly interact with the polymer matrix to impede the access
361 of pollutants to the bacterial cells and further increase the toxic resistance of the activated sludge
362 by retarding the contact of the metal with the bacteria within bioflocks. [37] However, once the
363 amount of released metal ions increased, the protective capacity of EPS to impede the access of
364 the CuO NPs to the activated sludge was weakened, due to their loose structure under high
365 toxicity condition. This explains the increased inhibition of CuO NPs to activated sludge at
366 higher concentrations observed in the present study. The toxicity of CuO NPs exposed to
367 bacteria can also be attributed to the changes of the sludge properties. [21] At low concentrations
368 of NPs, the dissolved Cu²⁺ ions from CuO NPs could function as the bridges between the
369 functional groups on the surface of bacteria and help to aggregate the microbes and promote the
370 bio flocculation formation. However, under higher concentrations of CuO NPs, the increased cell
371 surface charge weakened the strength between EPS and cations, resulting in the deterioration of
372 the flocculating ability of activated sludge. Moreover, it has been proven that the toxicity of CuO

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

375

376 **Conclusions**

377

378 In this study, the fate and behaviour of CuO NPs and copper ions in the waste activated sludge
379 process were investigated in SBR. The data indicate that the activated sludge process has the
380 potential to remove CuO NPs from wastewater. CuO NPs were efficiently retained by activated
381 sludge and CuO NPs were removed more effectively from the wastewater compared to copper
382 ions. Additionally, CuO NPs exhibited greater biosorption capacity and stronger affinity to
383 sewage sludge than copper salt. The short-term exposure to CuO NPs at 1 mg/L could cause
384 some effects on COD and ammonia removal. The exposure to CuO NPs and Cu²⁺ ions at higher
385 concentrations of 10 mg/L and 50 mg/L caused significant inhibition in biological wastewater
386 treatment. The results of bacterial integrity analysis imply that CuO NPs and copper salt at
387 higher concentrations reduced the viability of bacteria in the biological treatment process.

388

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390

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393

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501

502

503 **FIGURE CAPTIONS**

504

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

508

509 **Figure 2.** Kinetics of Cu²⁺ released from CuO NPs (A) and Cu²⁺ released from CuSO₄ (B). Error
510 bars represent standard deviations of triplicate measurements.

511

512 **Figure 3.** COD concentrations in the effluent of A) CuO NPs treatment; and B) CuSO₄ treatment.
513 Error bars represent standard deviations of triplicate measurements.

514

515 **Figure 4.** NH₄-N concentrations in the effluent of A) CuO NP treatment; and B) CuSO₄
516 treatment. Error bars represent standard deviations of triplicate measurements.

517

518 **Figure 5.** Cu²⁺ concentrations in the biosolids for A) CuO treatment; and B) CuSO₄ treatment.
519 Error bars represent standard deviations of triplicate measurements.

520

521 **Figure 6.** SEM images of activated sludge after CuO NPs and Cu²⁺ ions exposure at the
522 concentration of 10 mg/L after 5 h. A) Sludge in the control; B) Sludge in the treatment exposed
523 to CuO NPs; and C) Sludge in the treatment exposed to Cu²⁺ ions; D) EDS spectra for A); E)
524 EDS spectra for B); and F) EDS spectra for C).

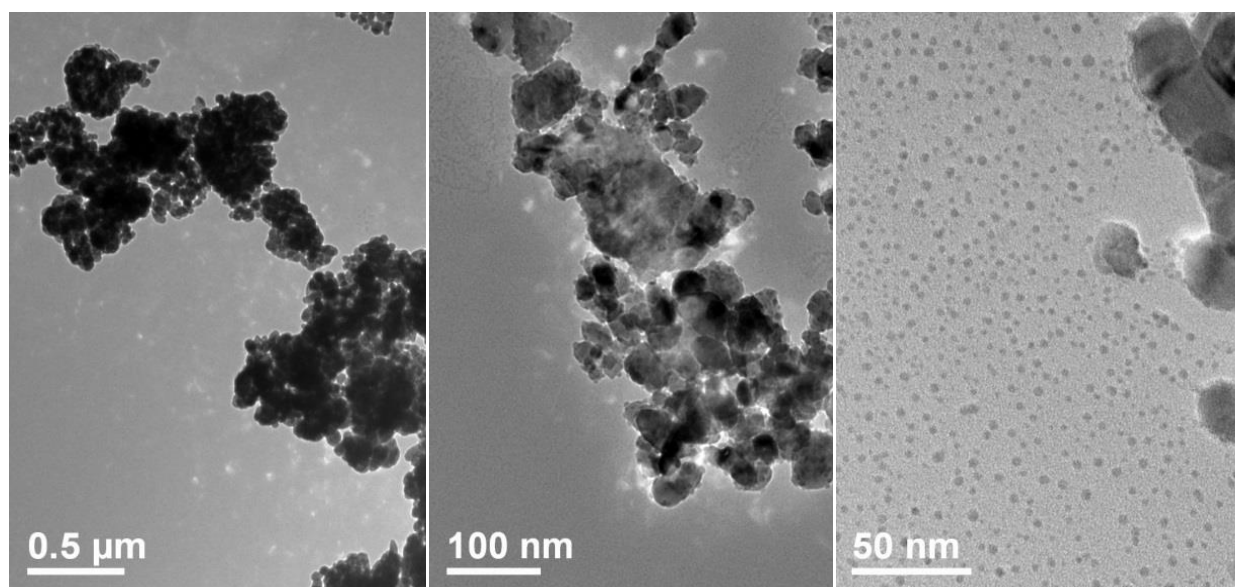
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526 **Figure 7.** Bacterial viability in A) control treatment; B) in activated sludge exposed to CuO NPs
527 at the concentration of 50 mg L⁻¹; and C) in activated sludge exposed to CuSO₄ treatment at the
528 concentration of 100 mg L⁻¹ at the end of the experiment using confocal microscopy.

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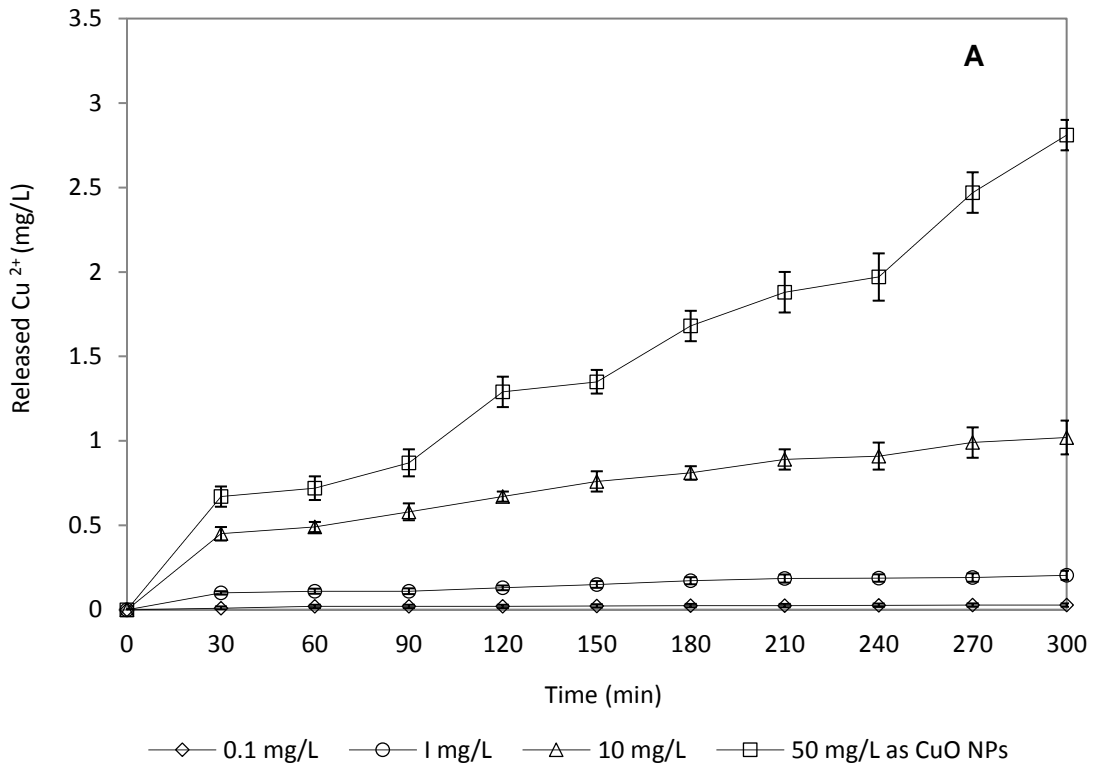
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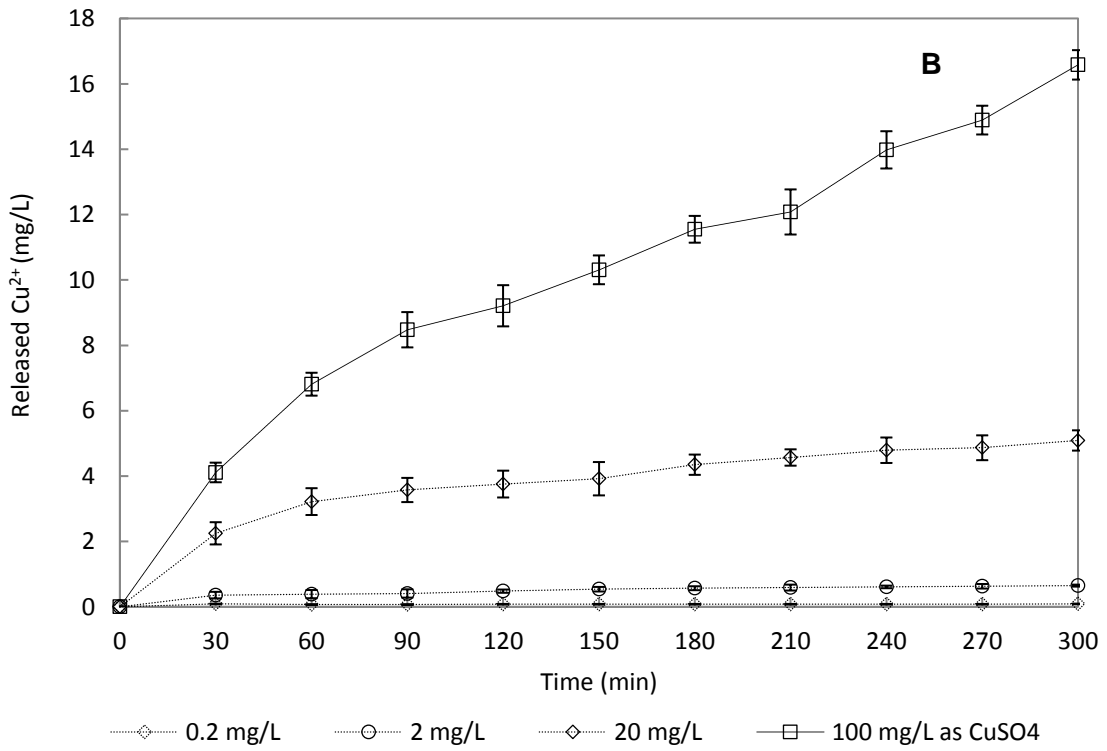
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533 Fig. 1



534

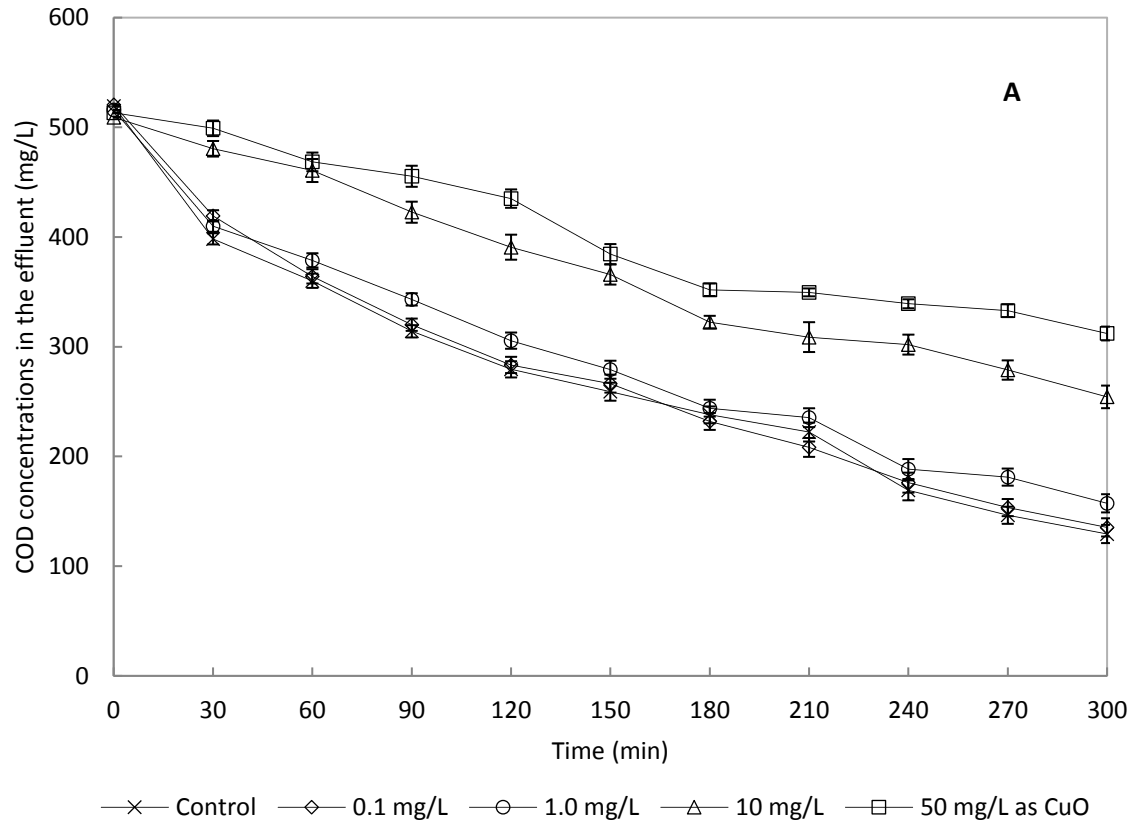
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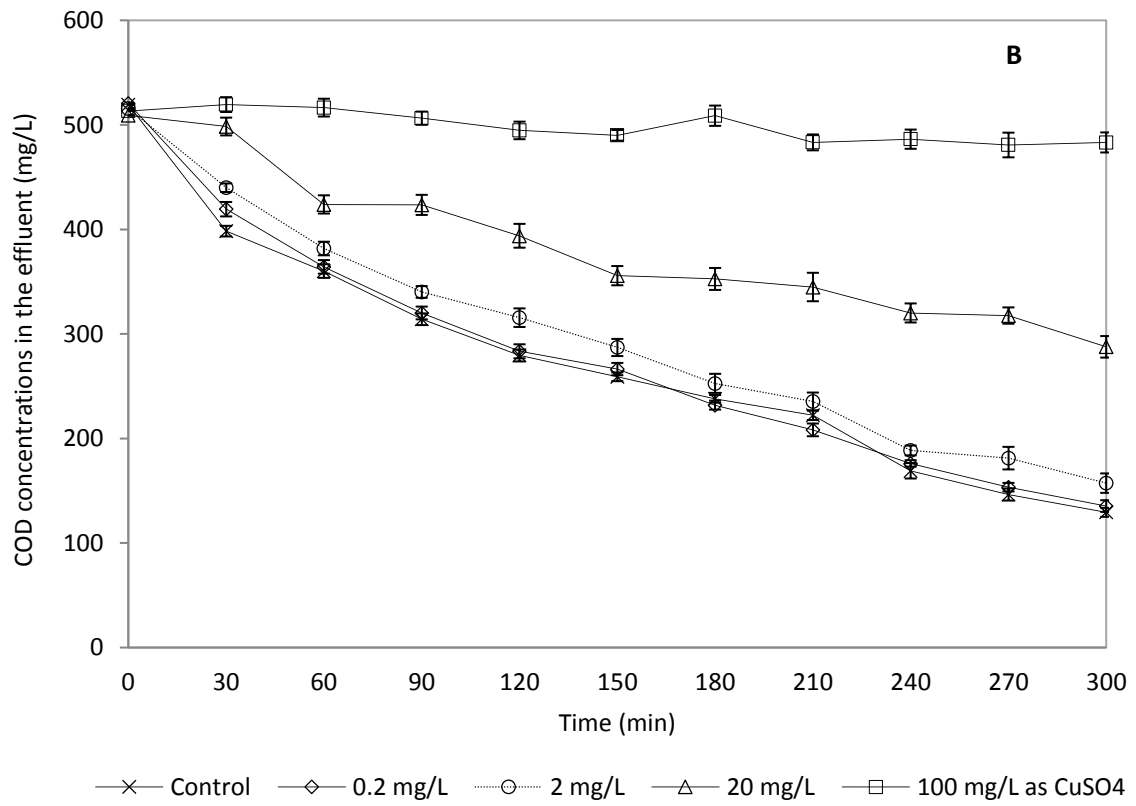
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537 Fig. 2

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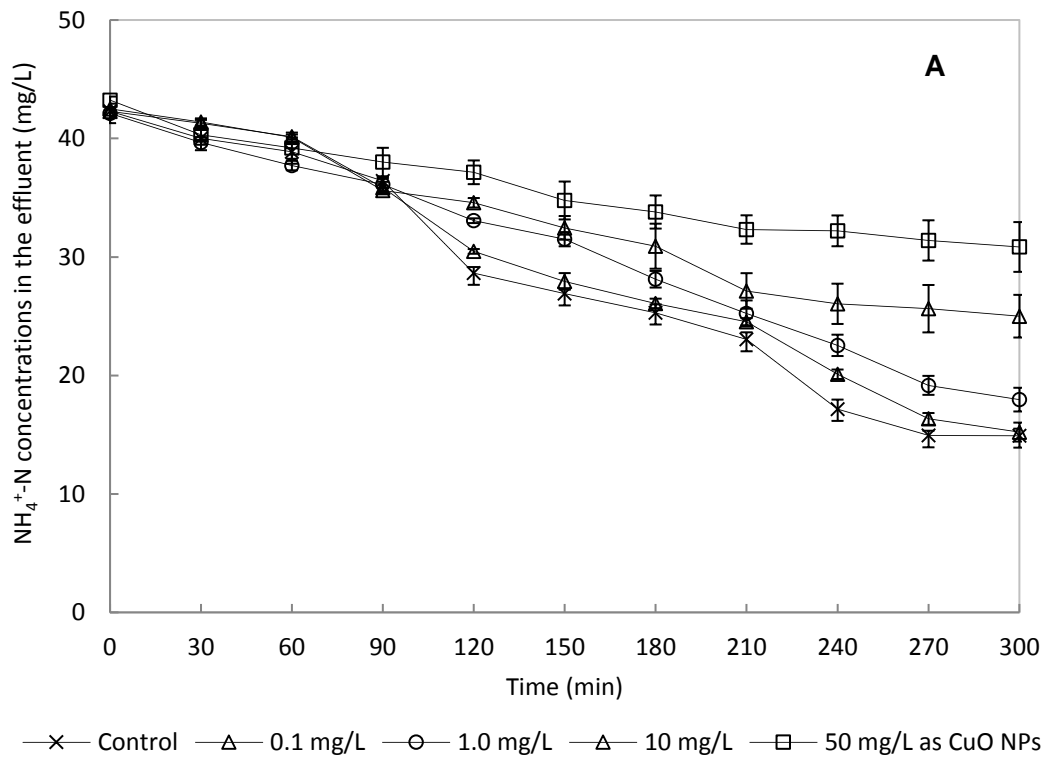


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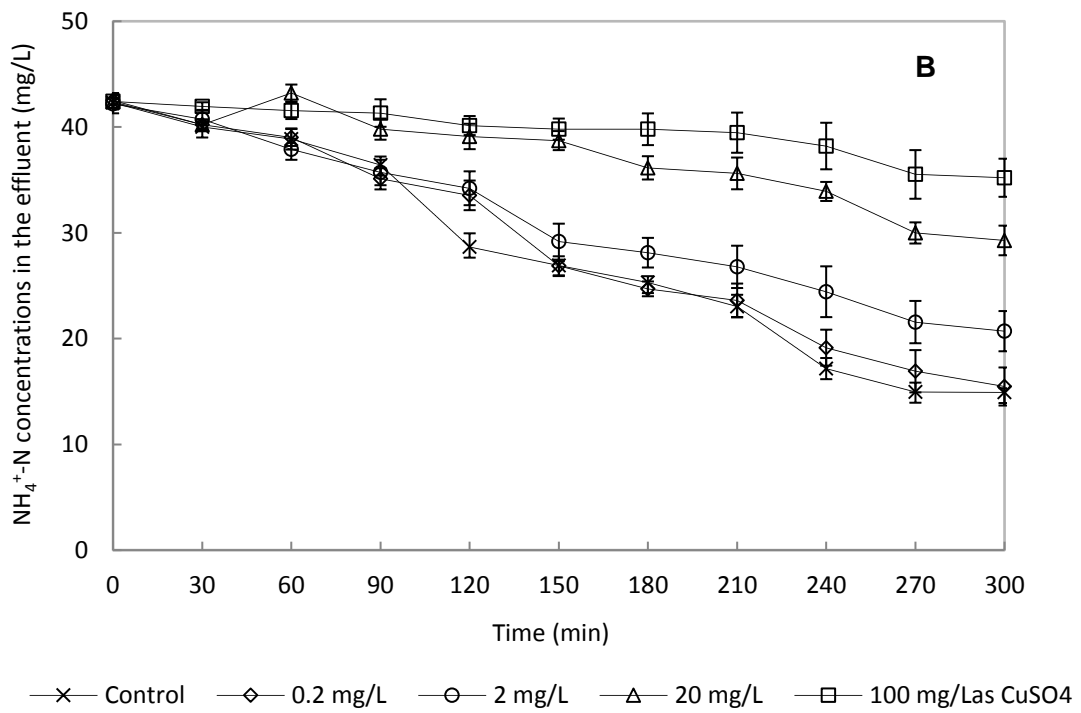
Fig. 3

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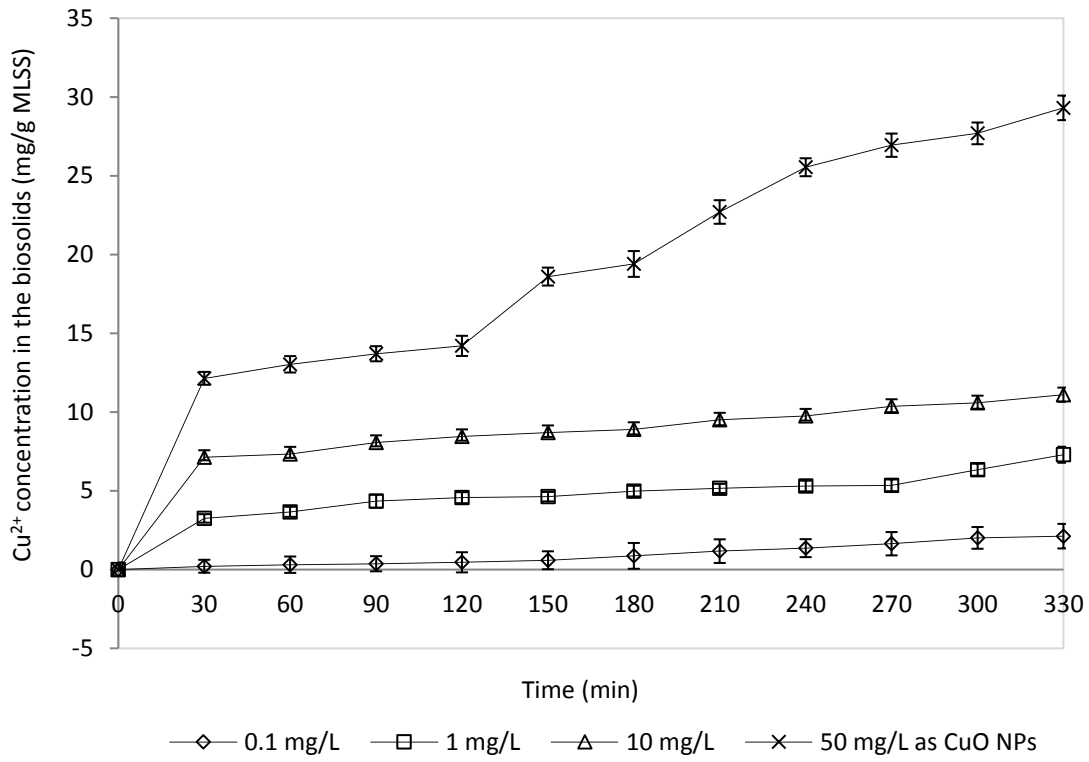
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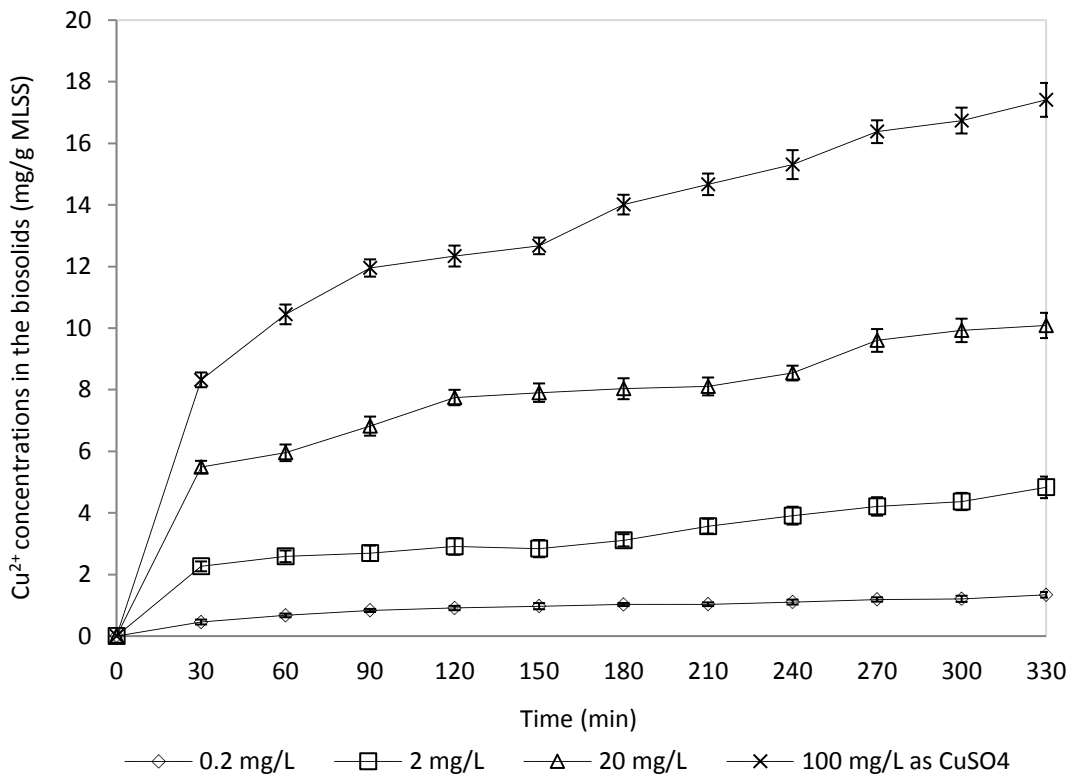
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546 Fig. 4

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548



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Fig. 5

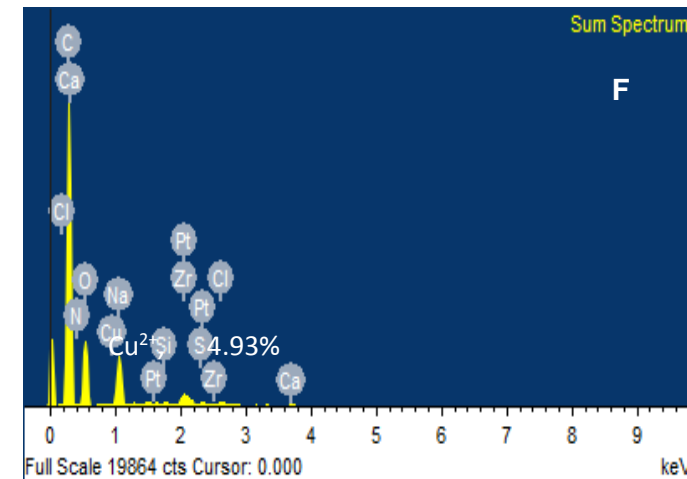
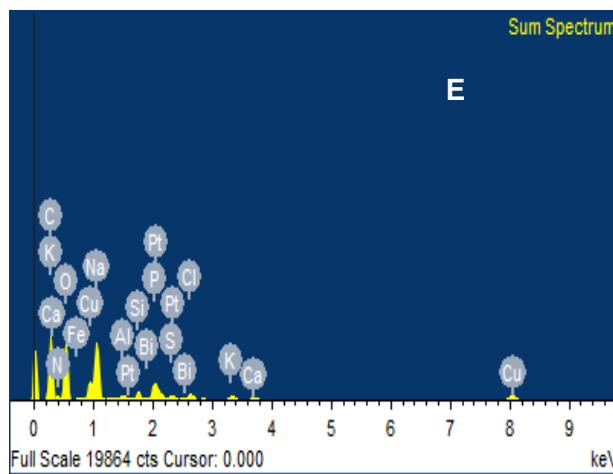
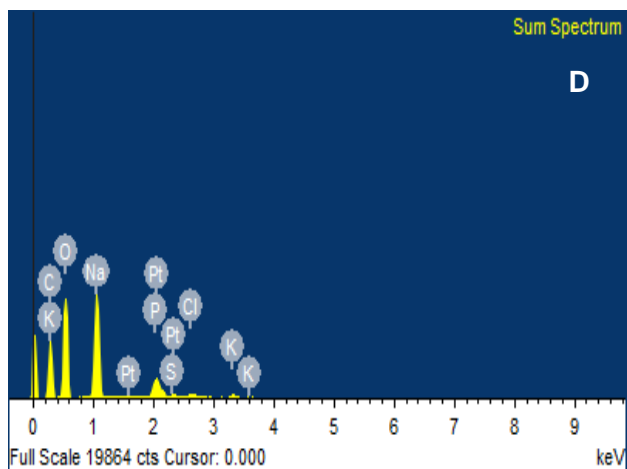
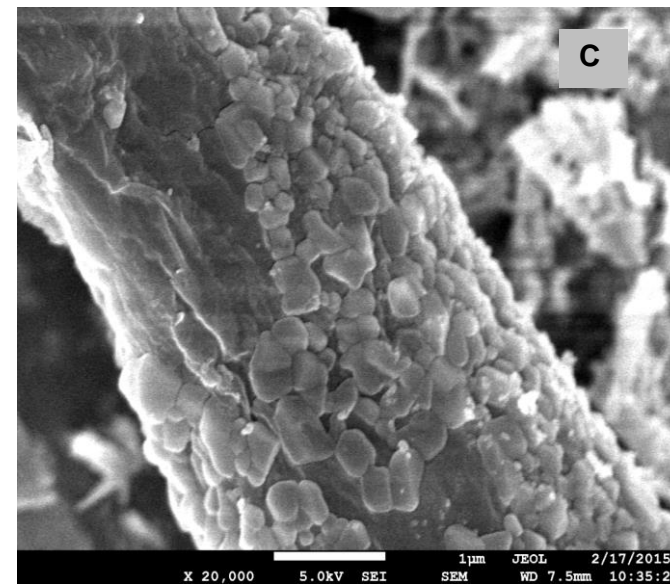
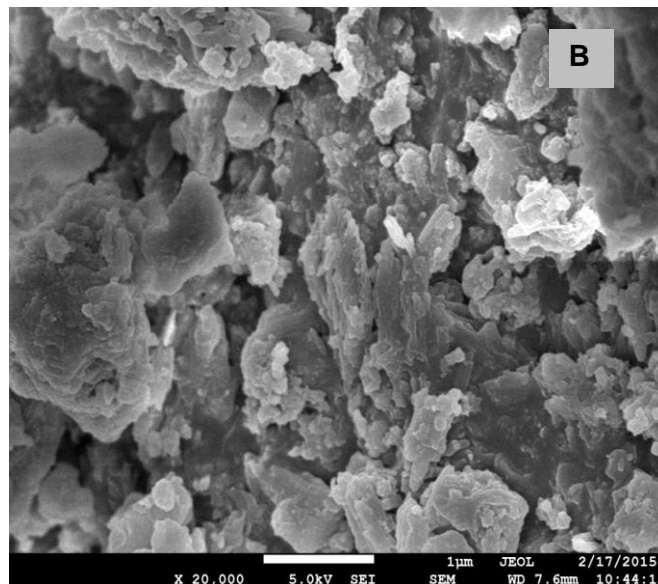
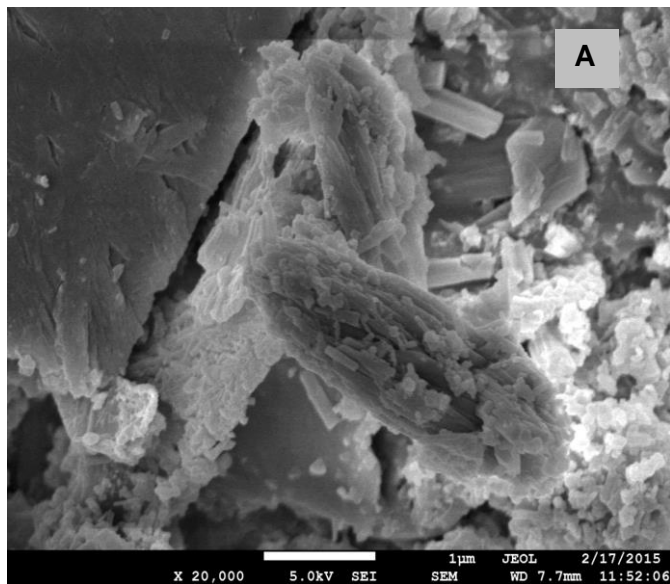
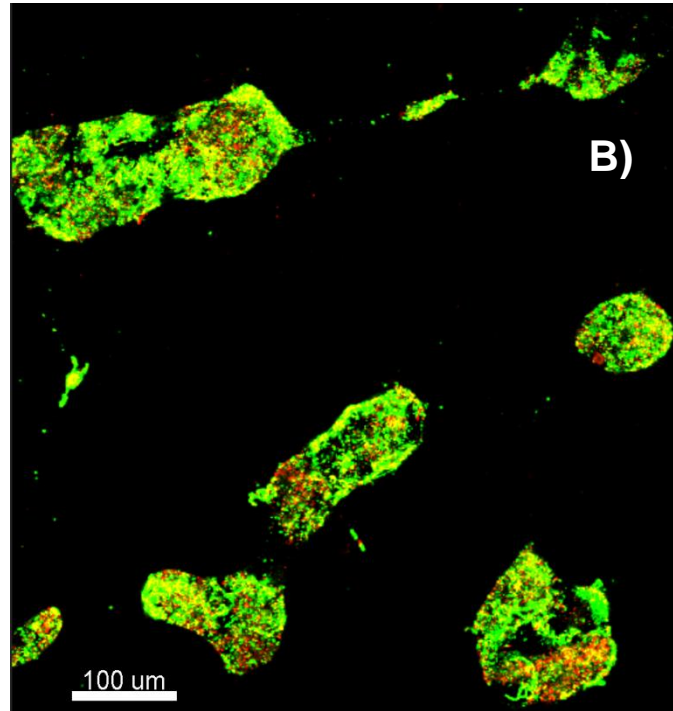
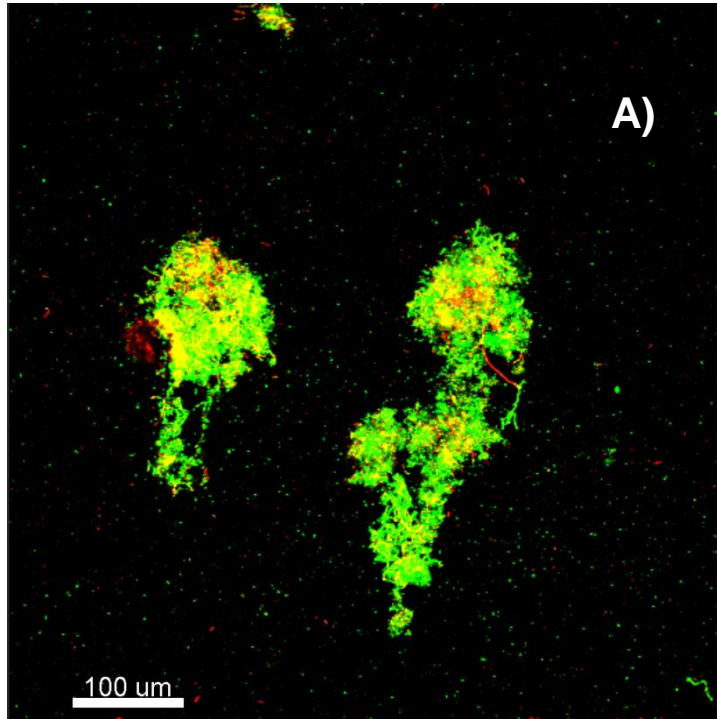
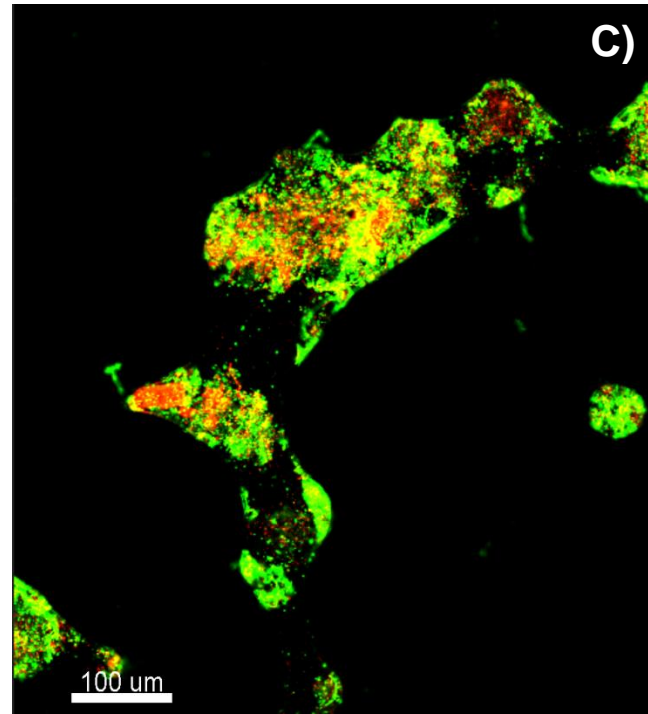


Fig. 6





551

552 Fig. 7

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