

1 **Fate and behavior of ZnO and Ag engineered nanoparticles and their**  
2 **bacterial viability assessment in a simulated wastewater treatment**  
3 **plant**

4  
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15  
16 **Abstract**

17  
18 Zinc oxide (ZnO) and metallic silver (Ag) engineered nanoparticles (ENPs) are  
19 increasingly used in consumer nanoproducts (e.g. paints, sunscreens, etc.) to meet  
20 customers' needs as well as in industrial applications (e.g. remediation) to enhance  
21 processes performance. As a result, ENPs will ultimately be released into the natural  
22 environment as well as engineered systems (e.g. wastewater treatment plants  
23 (WWTPs)). Here, we report findings on the fate, behaviour, and bacterial  
24 viability assessment of ZnO and Ag ENPs in a simulated WWTP fed with municipal  
25 wastewater. The ENPs were introduced at a dose rate of 0.83 mg/min for 240-hr to a 3L  
26 bioreactor (simulated WWTP) with hydraulic residence time (HRT) of 6 hr. The  
27 stability of the ENPs was found to be dependent on their dissolution and aggregation at  
28 different pH, where ZnO exhibited the highest dissolution at low pH compared to Ag  
29 ENPs. Also, the results showed that both ENPs had high affinity for the sewage sludge  
30 as they undergo aggregation under typical wastewater conditions. Results of effluent  
31 monitored daily showed mean COD removal efficiencies of  $71 \pm 7\%$  and  $74 \pm 8\%$  for  
32 ZnO and Ag ENPs in test units, respectively. The treated effluent had low mean  
33 concentrations of Zn ( $1.39 \pm 0.54$  mg/L) and Ag ( $0.12 \pm 0.06$  mg/L), however, elevated

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34 mean concentrations of Zn ( $54 \pm 39$  mg/g dry sludge) and Ag ( $57 \pm 42$  mg/g dry sludge)  
35 were found in the sludge –suggesting removal of the ENPs from the wastewater by bio-  
36 sorption and bio solid settling mechanisms. Using transmission electron microscopy  
37 (TEM), scanning electron microscopy (SEM), and X-ray diffraction (XRD); the  
38 morphologies of ZnO and Ag ENPs in the sludge from the test units were found  
39 comparable to those of commercial ENPs, but larger due to agglomeration. The bacterial  
40 viability assessment after exposure to ENPs using Live/Dead BacLight kit, though was  
41 not quantitatively estimated suggested, high resilience of the bacteria useful for  
42 biodegradation of organic material in the simulated wastewater treatment system.

43 **Keywords: Engineered nanoparticles, Stability, Removal, Wastewater, Bacterial**  
44 **viability**

45

## 46 **1. Introduction**

47

48 Nanotechnology is characterized by diverse commercial and industrial applications as  
49 well as widespread use of engineered nanoparticles (ENPs) which has led to their  
50 ultimate release into the environment at various stages of their life cycle <sup>[1]</sup>. Engineered  
51 technical systems, for example, WWTPs have the potential to act as major point sources  
52 of ENPs <sup>[1-2]</sup> to the aquatic environment <sup>[3]</sup> owing to inadequate treatment of the effluent  
53 <sup>[4-5]</sup>. This is of concern as toxicity studies have shown that ENPs can cause adverse  
54 effects to the microbial populations; including those useful for effluent treatment in the  
55 WWTPs <sup>[6-7]</sup>. On the other hand, reports have highlighted the effects of ENPs in the  
56 aquatic systems as largely influenced by fate and behaviour processes <sup>[8-10]</sup>. Therefore, to  
57 elucidate the potential impacts and implications of ENPs for safe and efficient operation  
58 of WWTPs, require an understanding of the nanoscale pollutants' fate and behaviour in  
59 such systems.

60

61 Several studies have reported the removal of ENPs using batch experiments from  
62 WWTPs. For instance, the ENPs where their partition in wastewater has been reported  
63 include; silver (Ag) (9,11,12), copper (Cu) (13), titanium dioxide (TiO<sub>2</sub>) (9; 14;  
64 15), Cerium oxide (CeO<sub>2</sub>) (5), silica (SiO<sub>2</sub>) (9), fullerenes (9), and zinc oxide (ZnO) (10).  
65 Taking into account the large diversity of ENPs even of the same “species” (16), studies  
66 reported until now on the removal of ENPs in the wastewater are too few to draw  
67 conclusive inferences on the effects and risks to WWTPs. Therefore, the goal of this

68 work is to improve our understanding on the fate and behaviour of ZnO and Ag ENPs.  
69 The choice of the two ENPs is because they are widely used in consumer products and  
70 industrial applications, and as a result will ultimately be released into the environment in  
71 large quantities <sup>[1-2,17]</sup>. For example, ZnO ENPs are of considerable commercial interest,  
72 and widely used in catalysts, sunscreens, cosmetics, and coatings while the Ag ENPs are  
73 used in cosmetics, façade paints, textiles, and biocides.

74  
75 The stability of NPs controls their dissolution, aggregation, persistence and fate in  
76 aqueous environments, and therefore, directly impacts on their distribution and uptake  
77 by biological life forms <sup>[18-19]</sup>. Bian et al. <sup>[20]</sup> reported that the dissolution and  
78 aggregation behaviour of ZnO NPs of < 10 nm in aqueous environments is dependent on  
79 solution pH, concentration of natural organic matter (NOM), and ionic strength (IS).

80  
81 The aim of the study was to investigate fate and behaviour of short term exposure of  
82 ZnO and Ag ENPs to wastewater during a simulated wastewater treatment process as  
83 well as assess bacterial viability, and then compare the results to other published  
84 literature on the treatment of these ENPs in wastewater treatment systems. More  
85 specifically the study objectives were to: (1) investigate the stability of ZnO and Ag  
86 ENPs in wastewater as a function of pH; (2) evaluate the fate and behavior of ENPs  
87 upon short term exposure to wastewater in simulated WWTP using ZnO and Ag ENPs  
88 as model NPs; and (3) assess and examine bacterial viability in wastewater upon  
89 exposure to ENPs.

## 91 **2. Materials and methods**

### 93 *2.1. Materials*

94  
95 Primary sewage (wastewater) was collected from Daspoort Wastewater Treatment Plant  
96 (Pretoria, South Africa: 25°44'03.72"S, 28° 10'40.32"E). The wastewater (*ca* 100 L) was  
97 collected just after the primary screening bar upon the removal of grit. Wastewater was  
98 collected once per week and stored in a cold room at 4°C to slow down bacterial  
99 activity, before it was fed into a simulated WWTP. ZnO and Ag ENPs were purchased  
100 from Sigma-Aldrich. HCl (32 % and density of 1.12), and NaOH pellets were purchased  
101 from Merck South Africa. All the chemicals used were of analytical grade.

102

### 103 2.2. *Engineered nanoparticles characterization*

104 ZnO and Ag ENPs were characterized before the start of the experiments. According to  
105 the manufacturer supplied data, the sizes for the ZnO and Ag ENPs were < 100 nm, and  
106 of spherical morphology. Characterization of the ZnO and Ag ENPs using high-  
107 resolution transmission electron microscope (TEM) revealed size distributions of 16–89  
108 nm and 29–50 nm, respectively. While ZnO ENPs consisted of a heterogeneous mixture  
109 of rods, cubes, regular and irregular spheres; the Ag ENPs had a homogenous spherical  
110 morphology. High-resolution transmission electron microscope (TEM) images of sludge  
111 exposed to ENPs were obtained using a JEOL JEM 2100F with a LaB6-cathode  
112 operated at a voltage of 200 kV (Tokyo, Japan), and high-resolution SEM measurements  
113 were made on FEI NovaNanoSEM230 operated at 2–5 keV (Tokyo, Japan). X-ray  
114 diffraction (XRD) measurements were carried out on a PAN Analytical X'Pert PRO PW  
115 3040/60 X-ray diffractometer (Almelo, Netherlands) equipped with a Cu K $\alpha$  wavelength  
116 of 0.154184 nm operating at 45 kV and 40 Ma. The spectra were collected in reflection  
117 geometry at 2 $\theta$ -values ranging from 10–80° with a step size of 0.02°.

118

### 119 2.3. *Simulated wastewater treatment plant layout*

120

121 A model wastewater treatment plant was constructed in accordance with the OECD  
122 design guidelines (21). The model comprised of two chambers: a stirred tank reactor and  
123 a clarifier simulating biological treatment using activated sludge system (see Fig. S1).  
124 Two model units, a test unit and a control unit, were run concurrently for parallel  
125 exposure experiments for each ENP type. Wastewater was fed using Watson-Marlow  
126 120S/DV pumps (Falmouth, Cornwall, UK) into the test and control units at 8.3 ml/min  
127 continuously for at least 168 hr and allowed to reach steady state. The steady state was  
128 established through monitoring the chemical oxygen demand (COD) removal. Upon  
129 reaching steady state conditions, wastewater containing ENPs (as a suspension) and no  
130 ENPs was also fed continuously using Watson-Marlow 120S/DV pumps (Falmouth,  
131 Cornwall, UK) into the pre-equilibrated test and control units for 240 hr. The selected  
132 exposure period was based on previous studies by Limbach et al. <sup>[5]</sup>. The high loading  
133 rate of ENPs used in the study, although not a truly representative of concentrations in  
134 typical wastewater from diffuse and point sources <sup>[15]</sup>, it was considered suitable for a  
135 short term test, for instance, in the event of undetected spillages and leakages from

136 storage facilities. Moreover, ENPs concentrations ranging from 100-1000 mg/L for  
137 CeO<sub>2</sub> have been used, without any acute toxicity to bacteria reported <sup>[5]</sup>.

138

### 139 2.3.1 *Simulated wastewater treatment plant operation*

140

141 The stirred tank reactor was aerated at 100 L/hr using compressed air introduced through  
142 a glass frit in a similar version as described by Limbach et al. <sup>[5]</sup>. A Watson-Marlow SCI  
143 Q 323S pump (Falmouth, Cornwall, UK) was used daily for 15 min to pump out sludge  
144 from the test and control units. The model unit design allowed for a hydraulic residence  
145 time (HRT) of 6 hr in the completely stirred tank reactor (aeration time). An HRT of 8  
146 hr (aeration time plus settling) has been considered typical for activated sludge systems  
147 and recently applied by Wang et al <sup>[9]</sup> in a sequencing batch reactor. Treated effluent and  
148 activated sludge samples (250 ml each) were collected daily from the clarifier of each  
149 unit. The sampling interval was based on the combined residence time of 12 hr in both  
150 vessels of each unit. As per the standard practice, part of the sludge collected from the  
151 clarifier was introduced as return activated sludge (RAS) to the completely stirred tank  
152 reactor using a Watson-Marlow SCI Q 323S pump for both the test and control unit.  
153 This was also meant to prevent wash-out of the sludge.

154

155 In this study, the chemical oxygen demand (COD) as well as Zn and Ag concentrations  
156 in treated effluent and sludge; were monitored over the 240 hr and the mean values and  
157 their variability were determined. The total Zn and Ag concentrations were measured  
158 using the ICP-OES and ICP-MS following the microwave acid digestion for the samples  
159 collected daily from the clarifier. The activated sludge collected was split into two equal  
160 parts, the waste activated sludge (WAS), and return activated sludge (RAS). The RAS  
161 was re-introduced into the aeration chambers daily to replenish the biomass. The WAS  
162 samples were used for analysis. In this study, only the WAS samples collected on the  
163 last day, following exposure for 240 hr, were characterized using TEM, SEM, and XRD  
164 to investigate the nature of the ENPs deposited on the sludge.

165

### 166 2.4. *Stability studies of engineered nanoparticles in wastewater*

167

168 To elucidate the fate and behavior of ZnO and Ag ENPs in wastewater, the stability of  
169 ZnO and Ag ENPs in wastewater was investigated in the pH range of 3–11 which covers

170 typical wastewater pH range of 7–8. Stock suspensions (1L) of ZnO and metallic Ag  
171 ENPs, each of concentration 100 mg/L were prepared. Nine aliquots (50 ml) were  
172 collected from each ENPs stock suspensions and their pH adjusted to be within the pH  
173 range 3–11 using HCl and/or NaOH. The aliquots were thoroughly mixed by gentle  
174 shaking and left to settle over 96 hr<sup>[5]</sup> before the Zn and Ag were measured in the  
175 supernatant using Inductively Coupled Plasma Optical Emission Spectroscopy (Jobin  
176 Yvon Ultima sequential ICP-OES, Longjumeau, France) to measure high concentration  
177 ranges, and Inductively Plasma Mass Spectroscopy (Agilent 7500CX ICP-MS,  
178 California, USA) for low concentration ranges.

179

### 180 2.5. *Bacterial viability assessments*

181

182 A portion of sludge (1 ml) collected from both the test and control units after 240 hr was  
183 used for bacterial viability assessments. The bacterial viability in each sample was  
184 analysed using L7007 LIVE/DEAD BacLight viability kit (Invitrogen, South Africa).  
185 The kit consists of two stains, namely: propidium iodide (PI) and SYTO<sup>®</sup>9. Both PI and  
186 SYTO<sup>®</sup>9 stain nucleic acids were used to differentiate between cells that were intact  
187 (live organisms – stained in green) and damaged cells (dead organisms – stained in red),  
188 respectively.

189

190 Ten µL aliquots of each stain were mixed together and 3 µL of the mixture was used for  
191 analysing 1 mL volume of bacterial suspension. The dye and bacterial suspension was  
192 mixed thoroughly and incubated at room temperature (~ 25<sup>0</sup>C) in the dark for 15  
193 minutes. Thereafter, 5 µL of the stained bacterial suspension was placed on a  
194 microscope slide and covered with a cover slip. The slides were examined under  
195 fluorescence microscopy using a BX51 microscope (Olympus, South Africa) fitted with  
196 an UPlanFl 100x/1.3 oil immersion objective. The excitation/emission maxima for the  
197 dyes were approximately 480/500 nm for SYTO<sup>®</sup>9 stain and 490/635 nm for propidium  
198 iodide. The images were captured and analyzed using a CC12 soft imaging system.

199

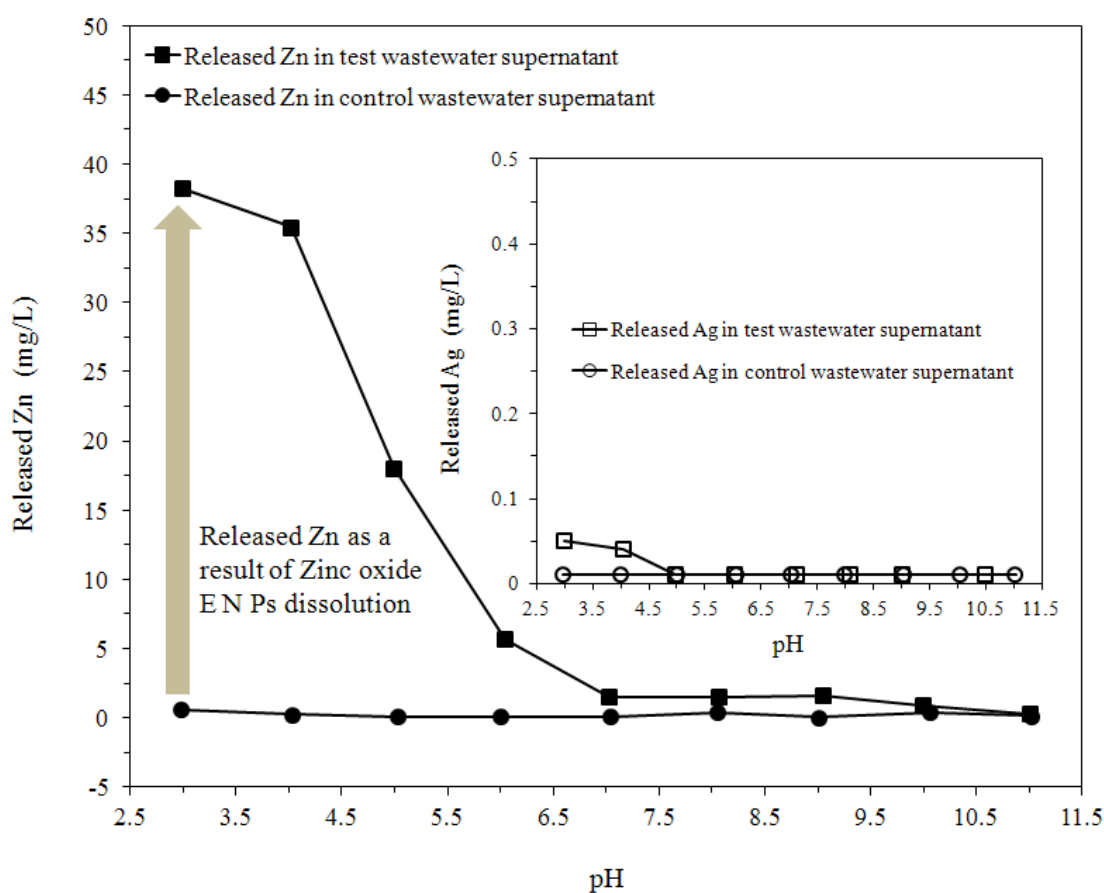
## 200 3. **Results and discussion**

201

### 202 3.1. *ZnO and Ag ENPs stability in wastewater - Effect of pH*

203

204 Establishing the stability of ZnO ENPs in wastewater is critical in order to elucidate  
 205 their dissolution, persistence, fate, and potential toxicity. For instance, Han et al. [22]  
 206 showed that the dissolution of bulk ZnO in an aqueous solution decreased with  
 207 increasing pH – and followed a direct interaction between bulk ZnO, hydrogen, and  
 208 hydroxyl ions in solution. In our study, the dissolution behaviour of ZnO ENPs in  
 209 wastewater appeared to follow similar chemistry as described by Han et al. [22]. In  
 210 particular, we observed significant ZnO ENPs dissolution under acidic conditions (pH 3  
 211 to 4), for instance, at pH 3, 38.2 mg/L Zn was measured in wastewater and this  
 212 corresponded to 47.5% of the total Zn added as ENPs (Fig. 1).  
 213



214 Figure 1 – pH dependence of ZnO and Ag (insert) ENPs stability in wastewater.

215

216 Furthermore, an increase in wastewater pH resulted to a rapid decrease in the total  
 217 amount of measured Zn, forming a plateau at pH values  $\geq 7.0$ . The rapid decrease in the  
 218 measured Zn as the pH increased is postulated as resulting from the removal of soluble  
 219 forms:  $Zn^{2+}$  and  $Zn(OH)^+$  from wastewater through hydroxide precipitation as the pH  
 220 conditions approach those typically found in wastewater (pH 7 – 8). Riechle et al. [23]

221 reported that zinc hydroxide ( $K_{sp} = 3.5 \times 10^{-17}$ ) as the dominant fraction of  $Zn^{2+}$  in an  
222 aqueous system within a pH range 7 – 12 at 25 °C. Once the insoluble zinc hydroxide is  
223 formed, both the hydroxide and undissolved ZnO ENPs are likely to be removed by bio  
224 sorption and bio solid setting mechanisms under typical wastewater conditions. Similar  
225 removal mechanisms have recently been postulated to account for the removal of both  
226 functionalized silver (fn-Ag) and  $TiO_2$  ENPs from wastewater<sup>[9]</sup>.

227  
228 Unlike in the case of ZnO ENPs, Ag ENPs exhibited limited solubility in wastewater  
229 and strong affinity for the sewage sludge over the entire pH range. Since no aeration of  
230 the wastewater was done during stability studies, it is expected that Ag ENPs did not  
231 undergo any dissolution in wastewater, and as such undissolved Ag ENPs were likely to  
232 have been removed from the wastewater by bio sorption and bio solid settling  
233 mechanism. However, under aerobic conditions of the simulated WWTP, it is possible  
234 that Ag ENPs can undergo oxidation forming highly insoluble  $Ag_2S$  ( $K_{sp} = 6.0 \times 10^{-51}$ )  
235 or  $Ag_2PO_4$  ( $K_{sp} = 1.3 \times 10^{-20}$ ). Therefore, further investigation on the speciation of Ag  
236 ENPs in the simulated WWTP would be required, as both species are highly insoluble,  
237 and therefore, settled out with the sewage sludge. It is however noteworthy that  
238 uncoated commercial ENPs used in this study may exhibit different behaviour in  
239 wastewater as compared to formulations with functionalized surfaces thereby affecting  
240 dissolution upon contact with wastewater. For instance, in studies using  $nTiO_2$  ENPs,  
241 the behaviour and effects in aqueous media were found to be distinctively different for  
242 bare and functionalized forms<sup>[24]</sup>, while similar effects have been observed for n-Ag  
243 ENPs<sup>[9]</sup>.

244

### 245 *3.2 Simulated wastewater treatment plant*

246

247 The pre-equilibrated simulated WWTP showed stable dissolved organic content,  
248 referred to herein as chemical oxygen demand (COD) in the wastewater after at least  
249 168 hr, with mean COD removal efficiencies of  $72 \pm 8$  % and  $74 \pm 9$  % for ZnO and Ag  
250 ENPs test units, respectively. The ZnO and Ag ENPs dispersions in wastewater were fed  
251 without interruptions into the completely stirred tank reactors. Also, the feed and treated  
252 effluent were collected and analyzed for COD, and the mean values are summarized in  
253 Table 1.

254



255 Table 1 Mean values of COD in feed, treated wastewater, and removal efficiencies for  
 256 ZnO and Ag ENPs test and control units over 240 hr.

257

	Feed COD mg/L	Treated COD mg/L	COD Removal %
ZnO ENPs test	384 ± 118	110 ± 39	71 ± 7
ZnO ENPs control	383 ± 146	71 ± 13	80 ± 5
Ag ENPs test	376 ± 96	97 ± 42	74 ± 8
Ag ENPs control	418 ± 70	92 ± 41	78 ± 8

258

259 The mean COD removal efficiencies in the test and control units after exposure to ZnO  
 260 ENPs dispersions over 240 hr were 71 ± 7 % and 80 ± 5 %, respectively. Similarly, the  
 261 mean removal efficiencies of COD for Ag ENPs in the test (74 ± 14 %) and control (78  
 262 ± 8 %) units were also determined. When operating efficiently, high-rate activated  
 263 sludge systems; the effluent would contain < 60 mg/L of COD <sup>[25]</sup>. Both the test and  
 264 control effluents had a COD content of 110 ± 39 and 71 ± 13 mg/L respectively for the  
 265 ZnO ENPs, which were both slightly above 60 mg/L. Similar results were observed for  
 266 Ag ENPs where COD content of 97 ± 42 and 92 ± 41 were recorded for test and control  
 267 units, respectively. Generally, the COD content in test units was slightly higher than in  
 268 the control units. However, the fact that comparable COD removal efficiencies were  
 269 observed for the test and control systems for both ZnO and Ag ENPs as shown in Table  
 270 1, suggests that the ENPs had negligible effects on ability of the wastewater bacteria to  
 271 biodegrade organic material, as measured by COD removal under the conditions of this  
 272 study. In this regard, the observed accumulation of Zn and Ag in the respective test units  
 273 sludge did not significantly impact on COD removal efficiency which was quite  
 274 comparable to the controls COD removal efficiency, where insignificant Zn and Ag  
 275 accumulated in the sludge (Fig. 2). Moreover, both ZnO and Ag ENPs test units  
 276 exhibited similar COD removal efficiencies of 71 ± 7 and 74 ± 8 mg/L respectively,  
 277 suggesting that the impact of these two ENPs on wastewater bacterial population over  
 278 short term exposure at high ENPs loadings are more or less similar.

279

280 3.2.1. Nanoparticles accumulation in the sludge

281

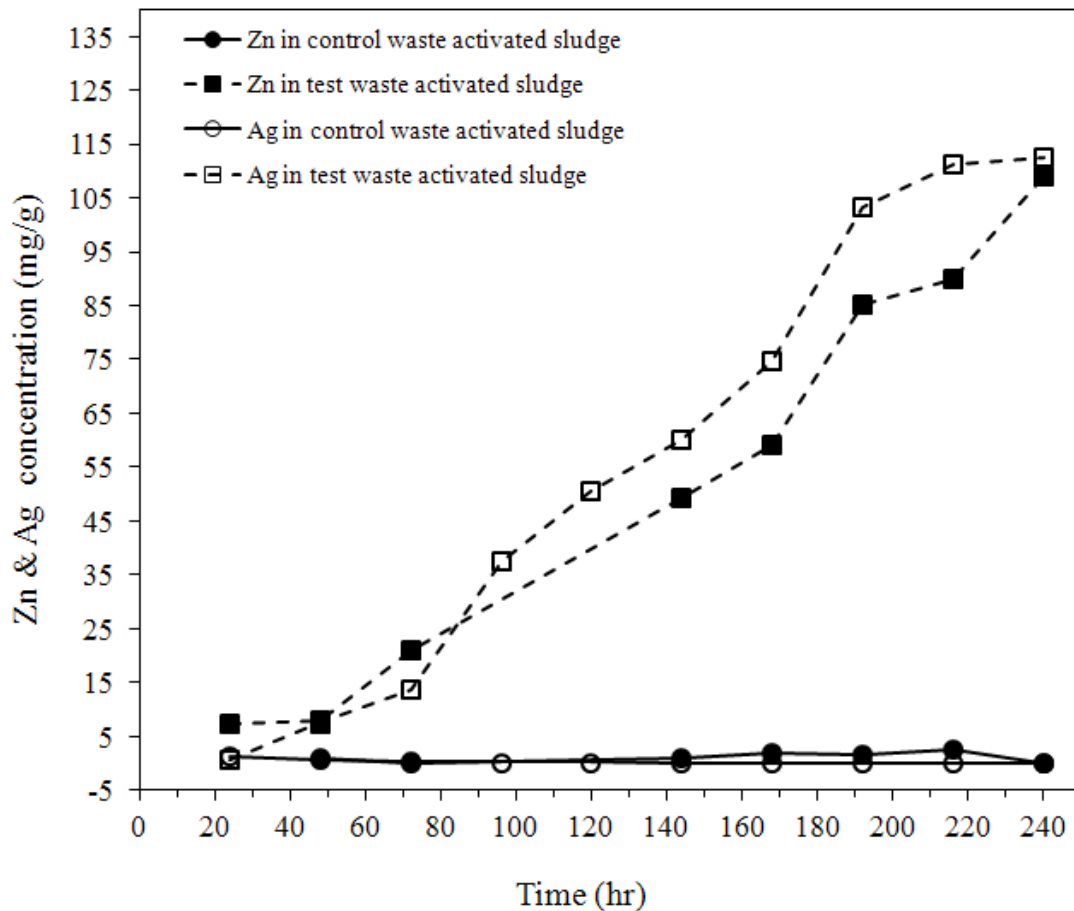
282 Scanning electron micrographs (SEM) of sludge collected after 240 hr of exposure to  
283 ZnO and Ag ENPs dispersions did not reveal the presence of individual ENPs-type  
284 crystals; probably because they were embedded in the sludge. However, the  
285 accumulation of both ZnO and Ag ENPs in the sludge (Fig. 2) was confirmed after  
286 measuring the concentrations of total Zn and Ag – based on dry weights determined  
287 using the solid digestion process. The results depicted gradual increase in the  
288 concentrations of Zn and Ag in the sludge over time in the test units as compared to  
289 concentrations in the control units where the values were virtually constant over 240 hr  
290 (Fig. 2). In the test units, the mean concentrations of Zn and Ag in the sludge were  
291 found to be  $54 \pm 39$  mg/g and  $57 \pm 42$  mg/g, respectively. Notably the high mean Zn  
292 and Ag concentrations in the sludge as compared to those found in the treated effluent  
293 ( $1.39 \pm 0.54$  and  $0.12 \pm 0.06$  mg/L) indicated strong affinity of ENPs to sewage sludge  
294 suggesting their removal from the effluent potentially through bio sorption and bio solid  
295 settling mechanisms. The incorporation of a significant portion, if not all, of the Ag  
296 ENPs into the settled bio solids resulting from poor dissolution of Ag ENPs in  
297 wastewater have been recently reported <sup>[9]</sup>. Although our study focused on short term  
298 ENPs exposure to wastewater at high ENPs loadings, while previous studies <sup>[9]</sup> focused  
299 on long term exposure using environmentally relevant loadings, similar ENPs removal  
300 mechanism by wastewater sludge would be expected.

301

302

303

304



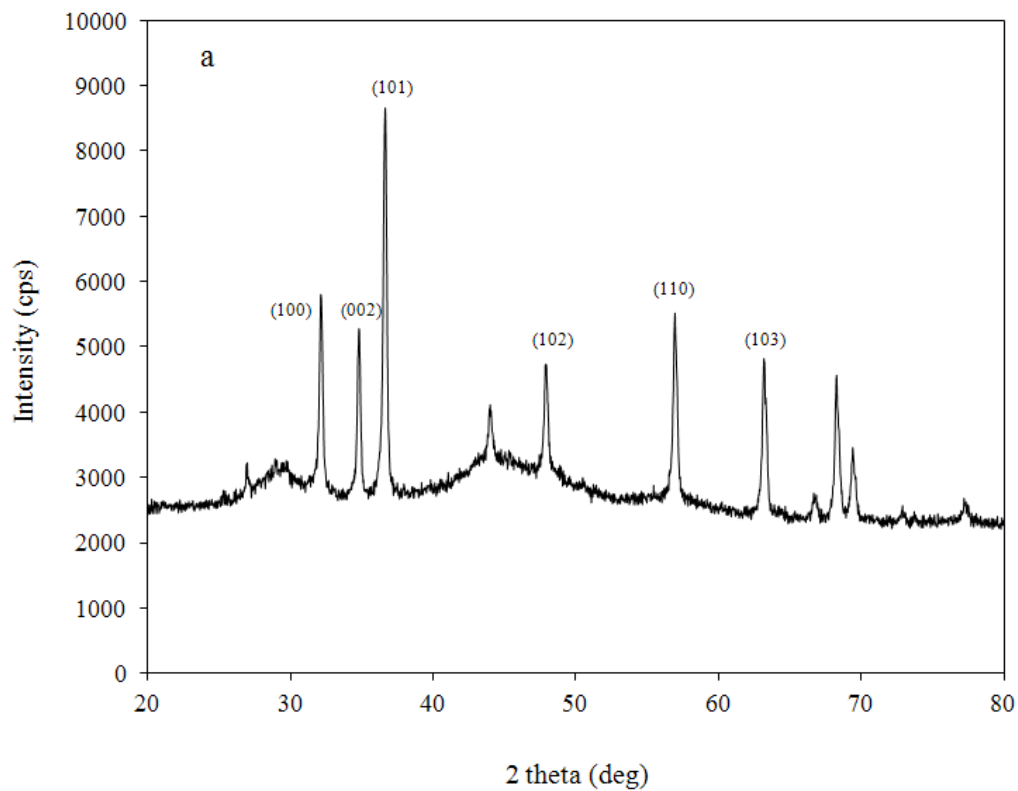
305

306 Figure 2 – Measured Zn and Ag concentrations in control and test waste activated  
 307 sludge over 240 hr.

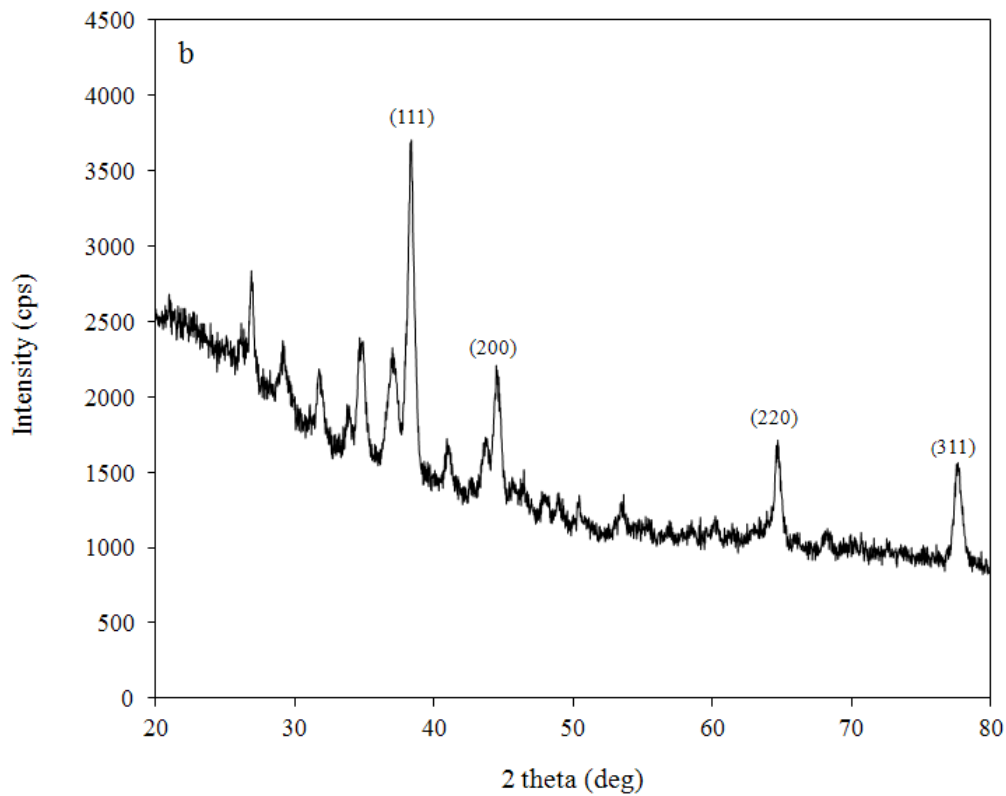
308

309 The XRD spectra obtained from the WAS collected after 240 hr following exposure to  
 310 ZnO and Ag ENPs are shown in Figs. 3a and b, respectively. The XRD spectra patterns  
 311 exhibited typical diffraction peaks of well-crystalline wurtzite structure for ZnO (Fig.  
 312 3a) and a face-centered-cubic form of metallic silver (Fig. 3b). The diffraction peaks for  
 313 the WAS exposed to ZnO ENPs showed sharper peaks, particularly the 002 diffraction  
 314 peak characterised by significant similarities to those previously reported by Pacholski  
 315 et al. <sup>[26]</sup>, and consistent with one-dimensional nanorod formation of ZnO ENPs.

316



317

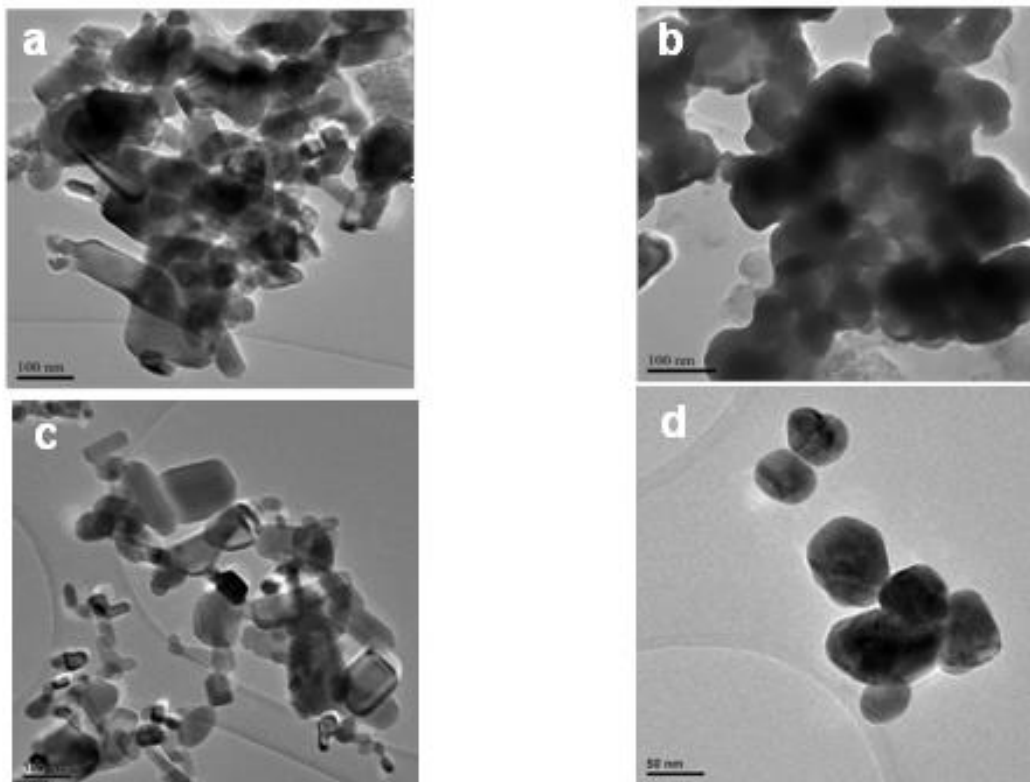


318

319 Figure 3 - X-Ray Diffraction (XRD) patterns for activated sludge exposed to (a) ZnO  
 320 and (b) metallic Ag ENPs.

321

322 The analysis of powder XRD pattern reveals the ZnO ENPs sample comprised of a



323 single phase, thus suggesting it was a phase pure material. However, an additional peak  
324 at  $2\theta$  value of 44 degrees is alien to the ZnO diffraction pattern, possibly arising from  
325 contamination by other metals commonly present in wastewater.

326

327 Figure 4 - Transmission electron micrographs (TEM) for activated sludge exposed to (a)  
328 ZnO and (b) metallic Ag ENPs. Micrographs (c) and (d) are for unexposed commercial  
329 ZnO and Ag ENPs respectively.

330

331 The sludge exposed to ENPs after 240 hr, was analysed using TEM, and the results for  
332 ZnO and Ag ENPs are summarised in Figs. 4a and b, respectively. The TEM images for  
333 ZnO and Ag ENPs before they were introduced into the sludge are given in Figs. 4c and  
334 d, respectively. Fig. 4c revealed the ZnO ENPs were polydispersed with an average size  
335 of 44 nm, size range distribution of 16–89 nm, and characterized by mixed morphology  
336 including rods, spheres, polygonal shapes, etc. For the Ag ENPs, the TEM findings  
337 showed uniform spherical morphology with narrow size distribution (29-50 nm) before  
338 they were introduced into wastewater (Fig 4d). For the ZnO ENPs, no distinctive  
339 differences between the micrographs before (average size 44 nm) and after exposure

340 (average size clusters of 70 nm) to the wastewater were noted, and in both cases  
341 appeared as loosely packed aggregates (Figs 4a and c).

342

343 However, in the case of Ag ENPs after exposure (Fig. 4b) large aggregates were formed,  
344 and it was difficult to isolate individual nanoparticles. Given tightly packed aggregates  
345 (Ag) (Fig. 4b) supposedly have fewer available surface sites compared to loosely packed  
346 aggregates (ZnO), however, no significant differences in COD removal efficiency  
347 (Table 1) and bacterial viability (Fig. 5) were observed over the short term exposure of  
348 wastewater to both ZnO and Ag ENPs. This observation was attributed to indifference in  
349 the concentrations of Zn and Ag in the activated sludge as illustrated by the results  
350 summarized in Table 2.

351

### 352 3.3. Bacterial viability assessment

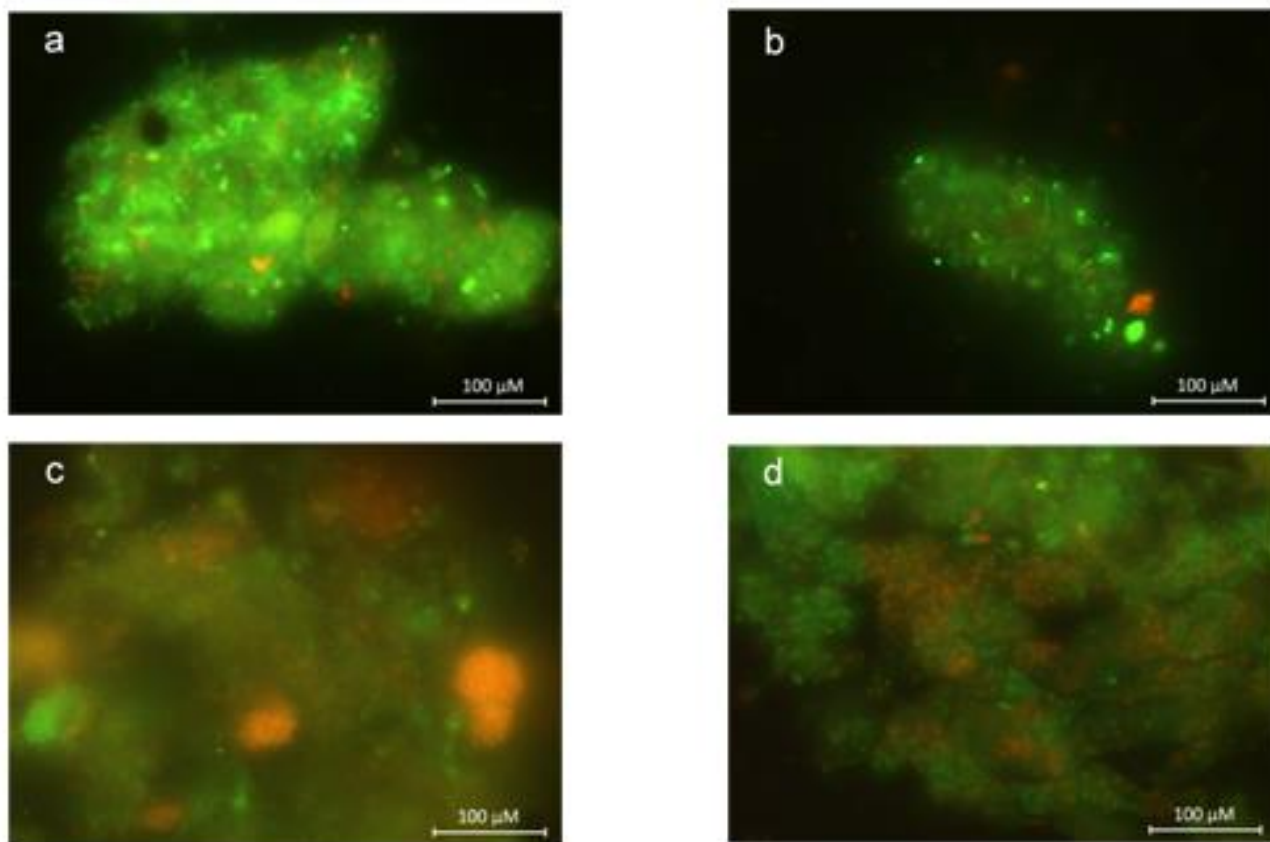
353

354 The LIVE/DEAD<sup>®</sup> BacLight bacterial viability kit technique was used to examine  
355 bacterial viability because of its distinctive advantage to visually discriminate between  
356 live and dead cells for a broad range of bacterial species found in wastewater. The  
357 results of the control and test units showed thick agglomerates of cells as evidence of  
358 floc formation (Fig. 5).

359

360 Although contingent of fluorescent red cells in test units spiked with ZnO and Ag ENPs  
361 (Figs. 5c and 5d, respectively) may indicate some bacterial cell membrane damage  
362 compared with the negative controls (Figs. 5a and 5b), a large contingent of fluorescent  
363 green cells are still evident in this system. This may have resulted in the observed  
364 negligible impacts on the ability of the wastewater bacteria to biodegrade organic  
365 material. Moreover, some cells with compromised membranes were also observed in the  
366 control units due to natural mortality. The ability of the activated sludge (wastewater  
367 bacteria) to remove COD and degrade organic material was not significantly hampered  
368 in the test unit. Figure 5 shows the bacterial viability in the control and test unit  
369 activated sludge flocs viewed by fluorescence microscopy, with live (green fluorescent)  
370 and dead (red fluorescent) bacterial cells. The wastewater system showed some  
371 resilience upon short term exposure to ZnO and Ag ENPs, – although no quantitative  
372 data (e.g., viability percentage, bio-volume per unit substrate, etc.) could be derived

373 using the Live/Dead BacLight kit because of the multiple layer complexity of flocs in  
374 the wastewater sludge and this is an area of future research focus.  
375 While most of the data reported until now on the antibacterial properties of ENPs were  
376 based on synthetic suspension systems, our results extend our knowledge in this field by  
377 providing additional insights and confirmation of such effects in typical wastewater.  
378



379  
380 Figure 5 - Bacterial viability in control activated sludge (a & b) and bacterial viability in  
381 activated sludge exposed to (c) ZnO ENPs and (d) Ag ENPs over 240 hr viewed by  
382 fluorescent microscopy.

383  
384 In summary, our results suggest that ENPs dissolution and aggregation in wastewater  
385 impacts on their stability, and both processes are influenced by environmental factors  
386 such as pH and ionic strength. Moreover, the wastewater bacterial cells appeared to be  
387 resilient to short term exposure of ZnO and Ag ENPs as observed during simulated  
388 wastewater treatment process. This was also supported by comparable COD removal  
389 efficiencies observed for both the test and control systems in this study, thus suggesting

390 negligible potential compromise of the useful wastewater bacteria upon short term  
391 exposure to these ENPs.

392

393 However, further studies examining quantitative resilience limits of specific bacteria  
394 and protozoa to ENPs exposure are needed, particularly over extended period of time to  
395 investigate possible bacterial adaptation, or potential of being comprised after certain  
396 time. Moreover, many older and smaller WWTPs employ fixed-film biological reactors  
397 (e.g. trickling filters) rather than the suspended biomass systems reported in this paper.

398 On that basis, further research on the removal of ENPs by attached microbial  
399 communities, is therefore needed. Additional research would also be needed to  
400 understand the possible release of ENPs from sludge incineration as well as their impact  
401 to soil where wastewater sludge is used for agricultural purposes.

402

#### 403 **4. Conclusions**

404

405 In this study, the fate and behaviour of ZnO and Ag ENPs as well as their bacterial  
406 viability in a simulated WWTP were investigated. This was achieved through evaluation  
407 of their stability in wastewater, monitoring of their interaction with activated sludge, and  
408 their eventual impact on bacterial population. Our findings revealed that a large  
409 percentage of the metal or metal-oxide ENPs showed strong affinity to the sewage  
410 sludge rather than dispersed in treated effluent. The deposition of ENPs on sludge is  
411 probably driven by bio sorption and bio solid settling mechanisms resulting in their  
412 limited dissolution. In this regard, ENPs are therefore likely to be introduced into the  
413 environment through use of sludge for agricultural purposes as well as possible release  
414 as fly ash during sludge incineration. The short term exposure of ZnO and Ag ENPs  
415 showed negligible effects on ability of the wastewater bacteria to biodegrade organic  
416 material in the simulated WWTP as evidenced by comparable COD removal both in  
417 test, and control systems. The overall adverse effects of ENPs to microbial population  
418 may have been limited by ENPs aggregation, probable shielding effects caused by  
419 extracellular polymeric substances (EPS) as well as inhibited bioavailability controlled  
420 by environmental factors (NOM, pH, etc.).

421

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423



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428

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