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Behaviour of titanium dioxide and zinc oxide nanoparticles in the presence of wastewater-derived organic matter and implications for algal toxicity

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33 **Abstract**

34 The properties of engineered nanomaterials (ENMs), such as their small size and increased
35 reactivity, enable their use in a wide range of products; however, these attributes may also pose an
36 environmental hazard. Wastewater effluent is expected to be a significant source of ENMs to the
37 aquatic environment. Little is known about the behaviour and effect of ENMs in this complex
38 matrix. The aim of this study was to assess the effect of titanium dioxide (TiO₂) and zinc oxide
39 (ZnO) ENMs to microalgae *Pseudokirchneriella subcapitata* in wastewater effluent and compare
40 with more commonly tested matrices, specifically Talaquil growth media and Suwannee River
41 humic acid. To better understand the toxicity results, ENM concentration, size and streaming
42 potential in the different experimental matrices were assessed. The different media types had a
43 significant influence on TiO₂ behaviour, with TiO₂ settling out of solution within the first 24 hours
44 in wastewater. However, as TiO₂ was not toxic to algae at the studied concentration, no difference
45 in effect was observed between media, humic acid and wastewater. In contrast, media type had less
46 of an influence on ZnO behaviour, with growth inhibition observed in all three media types at 72
47 hours, although the presence of organic matter delayed inhibition slightly. The results demonstrate
48 that organic matter properties can have a significant influence on ENM behaviour, and while this
49 did not translate into an effect on algae in the current study, it is still important to consider ENM
50 behaviour and fate when working in different matrices.

51

52 **Keywords:** engineered nanomaterials, wastewater, humic acid, algae, titanium dioxide, zinc oxide

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54

55

56 **Introduction**

57 Engineered nanomaterials (ENMs), such as zinc oxide (ZnO), titanium dioxide (TiO₂), silver
58 nanoparticles and carbon nanotubes, are increasingly used in areas such as energy and electronics,
59 as well as everyday consumer products including cosmetics, paints and clothing. The worldwide
60 annual production of TiO₂ was estimated to be up to 10,000 tonnes, while between 100-1,000
61 tonnes of ENMs such as ZnO and carbon nanotubes are expected to be produced per year.¹
62 Nanomaterials are defined² as having any external dimension or internal or surface structure in the
63 nanoscale, i.e., the size range from approximately 1 nm to 100 nm, and this means that they often
64 exhibit properties different from their bulk form. For example, ENMs have a larger surface area to
65 volume ratio than the bulk material, which can cause increased reactivity and this may increase the
66 formation of reactive oxygen species and cause oxidative stress in exposed organisms.³
67 Consequently, the same properties that have enabled ENMs to be applied in a wide range of
68 products may pose a toxicological hazard.

69 ENMs are expected to enter the environment during product manufacturing, use and
70 disposal.⁴ A number of studies have demonstrated that ENMs can be released from products
71 including clothing⁵ and painted facades.^{6, 7} Further, the use of ENMs in sunscreens and cosmetics
72 suggests that ENMs will reach wastewater treatment plants (WWTPs), with loads expected to
73 increase with greater ENM usage. Pilot and full scale WWTP studies indicate that the majority of
74 ENMs will be removed by conventional wastewater treatment processes (literature summarised in
75 Neale *et al.*⁸, Brar *et al.*⁹); however, some fraction of ENMs are still passing through WWTPs and
76 have been detected in wastewater effluent.^{10, 11} Due to difficulties with detecting low concentrations
77 of ENMs in complex matrices, much of the ENM exposure data comes from modelling studies. The
78 predicted levels in wastewater effluent range from nanogram per litre levels for ENMs such as
79 silver and carbon nanotubes to microgram per litre levels for more extensively used ENMs such as
80 TiO₂ and ZnO.^{12, 13}

81 In recent years, numerous studies have explored the effect of a range of ENMs on aquatic
82 organisms, including fish, crustaceans, bivalves and algae.¹⁴⁻¹⁶ Reported effects are wide ranging
83 and include mortality, malformation, growth inhibition, immobilisation and reduced reproductive
84 success. However, observed effects often occur at concentrations two to five orders of magnitude
85 greater than detected or expected ENM environmental concentrations.⁸ Further, the observed effect
86 can vary significantly depending on factors such as ENM preparation method, experimental
87 protocol and ENM coatings.¹⁷

88 The majority of ENM effect studies are conducted in pure water or media, with only a
89 limited number conducted in the presence of organic matter.^{18, 19} Organic matter, including humic
90 and fulvic acids, has been shown to alter the fate and behaviour of ENMs, altering stability and

91 leading to heteroaggregation,²⁰⁻²² or alternatively promoting disagglomeration.²³ Changes in
92 aggregation and/or agglomeration can have implications for toxicity. For example, the presence of
93 commercial humic acid changed the charge properties of TiO₂ ENMs, reducing physical interactions
94 between TiO₂ and the test organism, algae, and consequently decreasing TiO₂ toxicity.²⁴ In contrast,
95 Wang *et al.* showed that Suwannee River fulvic acid increased the toxicity of copper oxide (CuO)
96 ENMs to algae by reducing agglomeration/aggregation and facilitating increased release of free
97 copper ions.²⁵ Many studies in this area focus on reference organic matter, including International
98 Humic Substances Society standards, but, to the authors' knowledge, no studies have been
99 conducted in wastewater, with the exception of Kang *et al.* who studied the effect of carbon based
100 ENMs on microbial communities in wastewater effluent.²⁶ Wastewater-derived organic matter can
101 differ greatly from reference organic matter due to its microbial origin,²⁷ and this may influence
102 ENM behaviour and fate, which in turn can have implications for toxicity.

103 The aim of this study was to investigate the behaviour and effect of ENMs in wastewater
104 effluent. This was compared with experimental media commonly used for toxicity experiments and
105 reference humic acid. TiO₂ and ZnO ENMs were selected for this study as they are used in a wide
106 range of products and have been detected in wastewater effluent at microgram per litre levels.^{11, 28}
107 Further, ZnO ENMs can release free zinc ions,²⁹ while TiO₂ ENMs are relatively insoluble, and this
108 can influence toxicity. ENM behaviour was assessed by measuring concentration over time and
109 assessing changes in particle diameter and streaming potential, while growth and photosynthesis
110 inhibition of microalgae *Pseudokirchneriella subcapitata* was studied in the presence of ENMs.
111 Microalgae were selected as the test species as they represent the bottom of the aquatic food chain,
112 thus effects on algae could have implications for higher organisms.

113

114 **Materials and Methods**

115 *Chemicals*

116 All chemicals were of analytical grade. The herbicide terbutryn (Chem Service Inc, West Chester,
117 USA) was used as a positive control for the photosynthesis inhibition experiments. ZnCl₂ (Sigma
118 Aldrich, Castle Hill, Australia) was used as an experimental control in the ZnO experiments.
119 Suprapur nitric acid (65 %) (Merck-Millipore, Kilsyth, Australia) was used for inductively coupled
120 plasma mass spectrometry (ICP-MS) analysis.

121

122 *Engineered nanomaterials (ENMs)*

123 Two different ENMs were investigated in this study, TiO₂ and ZnO, all in dry powder form. The
124 TiO₂ sample was Aeroxide P25 (Sigma Aldrich, Castle Hill, Australia), which is a mix of the
125 crystallographic forms anatase and rutile (78 % anatase, 14 % rutile) and has a nominal particle

126 diameter of 21 nm (manufacturer's information). Two different ZnO samples (NM-110 and NM-
127 112) were studied, which consisted of materials recently analysed in an OECD project.³⁰ The
128 particle size distribution (PSD) of NM-110 ranges from 70 to 200 nm, with a mean size of 100 nm,
129 while NM-112 has a nominal PSD ranging from 30 to 50 nm. Further details about NM-110 and
130 NM-112 can be found in Singh *et al.*³¹

131 The ENM stock suspensions were prepared in algae growth media by stirring in a darkened
132 environment for approximately 18 hours. The concentration of the TiO₂ stock was 1000 mg L⁻¹, and
133 the concentrations of the ZnO stocks were 5000 mg L⁻¹, which were diluted to working stocks of
134 200 mg L⁻¹ (normalised to Zn concentration). Stirring was selected over other preparation methods
135 as it is considered more environmentally relevant.³² Sonication is often used for stock preparation,
136 but it can cause artefacts in toxicity testing.³³ The influence of preparation method, including
137 overnight stirring and sonication, on TiO₂ particle diameter is shown in Figure SI-1 of the
138 Supplementary Information. Freshly prepared stocks were used for each experiment to minimise the
139 effect of changes (e.g. aggregation/agglomeration, dissolution) during storage. An initial
140 comparison of the stability of the TiO₂ dispersions after different preparation methods was
141 performed by visual observation in wastewater, Talaquil growth media and ultrapure water
142 (18 MΩ cm) (Figures SI-2 to SI-4). These results indicated that the majority of the particles in
143 wastewater and Talaquil growth media sedimented out of suspension rapidly, whereas the sample
144 suspended in ultrapure water remained stable for much longer.

145

146 *Organic matter*

147 Sixty litres of secondary treated effluent were collected from a wastewater treatment plant in South
148 East Queensland (April 2013). On the day of collection, the water was filtered with a 1.6 µm glass
149 fibre filter followed by a 0.45 µm nylon filter. The pH, which was 8.1, was adjusted to 2 using HCl
150 and the water was stored at 4 °C in acid washed 20 L plastic containers for up to 8 months. Prior to
151 experiments the water was sterile filtered with a 0.22 µm polyethersulfone filter and readjusted to
152 the original pH using NaOH. The organic carbon concentration of the wastewater after 0.22 µm
153 filtration was 9.25 mg_C L⁻¹ and was analysed using an Analytik Jena Multi N/C 310. Further
154 wastewater characterisation can be found in Section SI-3 of the Supplementary Information.
155 Suwannee River humic acid (2S101H) (International Humic Substances Society, St Paul, US) was
156 selected as the reference organic matter.

157

158 *ENM Characterisation*

159 Laser diffraction

160 The particle diameter of TiO₂ and ZnO was assessed in the presence of growth media, humic acid
161 and wastewater using a Malvern Mastersizer 3000 (Malvern, UK). The wastewater experiments
162 were conducted in the presence or absence of *P. subcapitata* (1×10^4 cells mL⁻¹). For the data
163 analysis, a refractive index of 1.33 was used for the solutions, while refractive indices of 2.49 and
164 2.01 were used for the TiO₂ and ZnO ENMs, respectively. The samples were stirred at 2000 RPM
165 during the entire measurement cycle to ensure that the particles remained suspended and ENMs
166 were added until a suitable obscuration of the laser signal was reached (4 to 6.5 % depending on the
167 ENM type). Measurements were taken every 10 minutes over a period of an hour.

168

169 Streaming potential

170 To assess if the stability of the suspension could be further quantified, qualitative measurement of
171 the streaming potential of TiO₂ and ZnO ENMs in media, humic acid and wastewater at a fixed pH
172 was assessed using a Stabisizer PMX 200C (Microtrac Europe GmbH, Meerbusch, Germany). The
173 instrument can perform surface potential measurements of particles at higher concentrations and
174 larger sizes than electrophoretic mobility based techniques.³⁴ TiO₂ and ZnO ENM stock solutions
175 with a concentration of 10 mg mL⁻¹ were prepared for the streaming potential experiments. Prior to
176 each measurement, 1 mL of stock solution was added to 9 mL of media, humic acid or wastewater,
177 respectively. As there are no reference materials available for verification of the streaming potential
178 measurements, an undiluted suspension of commercially available zeta potential quality control
179 material (polystyrene latex particles, Malvern, UK), known to exhibit stable surface charge, was
180 also measured. A 200 µm gap piston was used and the streaming potential of each sample was
181 measured for 10 minutes, with data collected every 3 seconds.

182

183 Inductively coupled plasma mass spectrometry (ICP-MS)

184 The concentration of ENMs in media (TiO₂ only), humic acid and wastewater in the presence of
185 algae over 72 hours was analysed using an Agilent 7500 series ICP-MS. All samples were spiked
186 with an internal standard containing lithium, germanium, indium, beryllium, rhodium, yttrium and
187 scandium. A wastewater effluent certified reference material (BCR-713, European Commission,
188 Geel, Belgium) was also included for analysis. The limit of detection was 0.60 µg L⁻¹ for Ti and
189 0.64 µg L⁻¹ for Zn and the percent recovery of the spiked compounds in the working range (10-100
190 µg L⁻¹) ranged from 94-111%.

191 Highly insoluble Ti was digested using an ammonium persulphate method developed by
192 Khosravi *et al.*³⁵ Briefly, 2 mL of sample was collected from the supernatant at the top of the flask
193 at different time points and evaporated to dryness in porcelain crucibles on a hotplate, then covered
194 with 1 g of ammonium persulphate and fusion was performed using a Bunsen burner. The products

195 of the fusion were soaked in 2 % HNO₃ for 30 minutes and then made up to a final volume of
196 20 mL. Ammonium persulphate and wastewater controls were included.

197 Total Zn concentration in wastewater and humic acid over time was assessed using
198 microwave digestion. Briefly, 1 mL of sample was collected from the supernatant at different time
199 points and added to 4 mL of 65 % HNO₃. The sample was digested using the following microwave
200 program at 1400 W: ramp to 125 °C over 10 minutes, ramp up to 175 °C over 15 minutes, then hold
201 at 175 °C for 15 minutes. The sample was then diluted with ultrapure water to a final HNO₃
202 concentration of 2 %. Wastewater without ZnO was also digested as an experimental control.

203 ZnO dissolution to dissolved Zn ions in humic acid and wastewater over time was also
204 assessed by ICP-MS. Briefly, 4.9 mL of sample was filtered through a 0.1 µm PVDF filter and
205 0.1 mL of 65 % HNO₃ was added to give a final HNO₃ concentration of 1.3 %. While 100 nm is the
206 upper end of the nominal ENM size range, the laser diffraction results indicated that the size of ZnO
207 in solution was much larger than 100 nm (see Results and Discussion), thus the particulate ZnO
208 should be retained by the filter while dissolved Zn should pass through. Similar methods to assess
209 Zn dissolution have been applied previously by Franklin *et al.*²⁹ and Fabrega *et al.*³⁶

210

211 *Algae toxicity testing*

212 Green microalgae *P. subcapitata* was selected to assess growth and photosynthesis inhibition by the
213 studied ENMs. The algae were cultured in Talaquil growth media³⁷ in an incubator (22 °C,
214 90 RPM) under constant fluorescent lighting. The experiments were conducted in 100 mL
215 autoclaved Erlenmeyer flasks with 50 mL of Talaquil growth media, humic acid or wastewater. A
216 starting density of 1×10^4 cells mL⁻¹ was added to each flask. To facilitate algae growth,
217 concentrated humic acid stock was diluted in Talaquil growth media to achieve a final humic acid
218 concentration of 10 mg_C L⁻¹, while the wastewater was supplemented with 5 mL of 10 times
219 enriched Talaquil growth media. This diluted the wastewater concentration to 8.3 mg_C L⁻¹. For the
220 ZnO experiments the Talaquil media was prepared without ethylenediaminetetraacetic acid
221 (EDTA). Details on the differences in Talaquil media between TiO₂ and ZnO ENM experiments
222 can be found in Section SI-4. Concentration range finder experiments in Talaquil growth media
223 were conducted with TiO₂ concentrations from 0.1 to 10 mg L⁻¹ and ZnO concentrations from 0.05
224 to 2 mg L⁻¹ (Figures SI-6 and SI-7), with the majority of experiments in the study conducted at 1
225 mg L⁻¹ of TiO₂ and 0.1 mg L⁻¹ ZnO (normalised to Zn concentration). All experiments were
226 conducted with three replicates.

227 Algae growth was assessed using an Accuri C6 flow cytometer (BD, Franklin Lakes, US).
228 Due to the presence of chlorophyll, *P. subcapitata* is autofluorescent above $\lambda = 600$ nm; therefore,
229 fluorescence detector 3 ($\lambda > 670$ nm) was used and the algae peak was gated to separate it from

230 interference from ENMs or organic matter (example shown in Figure SI-8). Cell count was assessed
231 at 0, 24, 48 and 72 hours. The data were reported as growth inhibition relative to the control, which
232 was calculated using Equation 1:

$$\text{Growth Inhibition}(\%) = \frac{\text{Viable cells in sample} \left(\frac{\text{cells}}{\text{mL}} \right)}{\text{Viable cells in control} \left(\frac{\text{cells}}{\text{mL}} \right)} \times 100 \quad (1)$$

233

234

235 Photosynthesis inhibition was assessed using Maxi-Imaging-PAM (IPAM, Walz GmbH, Germany).
236 The photosynthetic yield (Y) of the samples and controls were measured at 24, 48 and 72 hours.
237 The data was reported as photosynthesis inhibition relative to the control and was calculated using
238 Equation 2:

$$\text{Photosynthesis Inhibition}(\%) = \frac{Y(\text{Sample})}{Y(\text{Control})} \times 100 \quad (2)$$

239

240

241

242 **Results and Discussion**

243 *Behaviour of TiO₂ ENMs in wastewater, media and humic acid*

244 The fate and behaviour of TiO₂ ENMs in the different matrices was assessed by monitoring
245 concentration over time and changes in particle size (due to agglomeration and/or aggregation) and
246 streaming potential. The concentration experiments were conducted under identical conditions as
247 the toxicity tests, while the additional characterisation experiments were conducted over shorter
248 time periods, but were used to provide further information about the behaviour of the ENMs in
249 media, humic acid and wastewater. The different experimental matrices had a dramatic effect on
250 TiO₂ ENM behaviour, with both ICP-MS analysis and laser diffraction indicating that TiO₂ ENMs
251 were not stable in wastewater. The results shown in Figure 1 A illustrate that after 24 hours in
252 wastewater the majority of Ti was lost from the supernatant, with the Ti concentration the same as
253 the wastewater background levels (Figure SI-9 A). In contrast, as little as 25 % of Ti was lost from
254 the supernatant over 72 hours in the presence of humic acid (Figure 1 A). The laser diffraction
255 results showed that the median TiO₂ particle diameter (D₅₀) increased rapidly over time in
256 wastewater, with the presence of algae further increasing particle diameter (Figures 2 A and SI-10).
257 In contrast, the TiO₂ appeared to be more stable in humic acid compared to media. Laser diffraction
258 has been previously used to assess ENM aggregation in the presence of organic matter.³⁸ Despite
259 the nominal nanometre size range of the ENMs, the laser diffraction analysis indicated that in all

260 studied samples, particles were likely to form agglomerates and/or aggregates in the micrometre
261 size range under the different experimental conditions (Figure 2). Results from Taurozzi *et al.*³⁹
262 indicate that significant amounts of ultrasonic energy is required to break up the agglomerates that
263 are likely to be present. As sonication was not applied in the current study, it was not possible to
264 differentiate between agglomerates and aggregates.

265 The lack of stability of TiO₂ ENMs in wastewater was further supported by the streaming
266 potential measurements. While the surface potential of TiO₂ ENMs in media and humic acid
267 remained relatively constant over the 10 minute measurement, there was a larger difference in
268 streaming potential for TiO₂ in wastewater, compared to wastewater alone, which indicates that the
269 TiO₂ ENMs were unstable and likely to aggregate (Table SI-2). Keller *et al.* explored the behaviour
270 of TiO₂ in a number of water sources and found that stabilisation was promoted by high organic
271 carbon content and low ionic strength.⁴⁰ In the current study the organic carbon concentration was
272 slightly higher in the humic acid experiments than in the wastewater experiments (10 vs. 8.3 mg_C L⁻¹).
273 Previous studies have suggested that humic acid can coat TiO₂ particles, increasing electrostatic
274 repulsion and preventing aggregation/agglomeration.²⁴ The organic carbon properties of wastewater
275 are very different to humic acid, with more low molecular weight neutrals and biopolymers,²⁷ and
276 these may have a reduced affinity for TiO₂. Further, the presence of ions in the wastewater may
277 shield the TiO₂ negative charge, leading to increased aggregation/agglomeration. The conductivity
278 of the wastewater was 2.3 mS cm⁻¹ (after pH adjustment), while conductivity of the algae growth
279 media was 1.0 mS cm⁻¹. The presence of divalent ions, such as calcium, has previously been shown
280 to have a significant influence on TiO₂ stability.⁴¹ Divalent ion concentrations were not measured
281 in the current study, but based on past analysis of wastewater effluent in Eastern Australia,⁴² the
282 concentration of divalent ions in wastewater is likely to be higher than in the other studied matrices.

283

284 *Behaviour of ZnO ENMs in wastewater, media and humic acid*

285 In contrast to the TiO₂ ENMs, both types of ZnO ENMs studied were more stable in wastewater,
286 with around 80 % of added Zn remaining in the supernatant after 72 hours (Figures 1 B). It was not
287 clear whether Zn was present in particulate or dissolved form in the ICP-MS experiments as only
288 total Zn was measured. NM-112 had a greater tendency to aggregate over time compared to NM-
289 110, particularly in the growth media experiment, and had a larger average particle size at time zero
290 (Figure 2 B and C). No significant change in surface potential was observed during the 10 minute
291 streaming potential measurements for the ZnO ENMs (Table SI-2), which is also reflected in the
292 results from the laser diffraction measurements at 10 minutes, where no discernible size difference
293 was detected (Figure 2). Singh *et al.* also found that NM-112 formed larger aggregates in deionised
294 water, seawater and *D. magna* media compared to NM-110 using differential centrifugal

295 sedimentation.³¹ The same study also found distinct differences in the zeta potential of the ENMs in
296 the different media types, but there was no significant difference between NM-110 and NM-112,
297 which agrees with the observations in the current study. It should be noted that the sample
298 preparation applied in Singh et al. included ultrasonication, which was not performed in the current
299 study.³¹

300 It should also be noted that due to the large dilution required after microwave digestion, the
301 ZnO ENM fate experiments were conducted at $20 \text{ mg}_{\text{Zn}} \text{ L}^{-1}$ (200 times higher than the toxicity
302 experiments), in contrast to the TiO_2 ENM fate experiments, which were conducted at the same
303 concentration as the toxicity experiments (1 mg L^{-1}).

304 ZnO ENM dissolution experiments in humic acid indicated that around 90 % of dissolved
305 Zn was released from both NM-110 and NM-112, with the majority of dissolution occurring within
306 the first 24 hours (Figure 1 C). The rate of ZnO ENM dissolution in algal toxicity experiments
307 varies substantially within the literature (e.g. 4-50 %) and can be influenced by media composition,
308 algae concentration, pH and ZnO ENM concentration.^{43, 44} For example, Zhao *et al.* found that ZnO
309 ENM dissolution increased with decreasing ZnO ENM concentrations in experiments with *Daphnia*
310 *magna*.⁴⁵ Many studies conduct dissolution experiments at concentrations many times higher than
311 the toxicity experiments, but in the current study, both the dissolution and effect studies were
312 conducted at $0.1 \text{ mg}_{\text{Zn}} \text{ L}^{-1}$. Zn dissolution was also assessed in wastewater, but the high wastewater
313 content appeared to interfere with the analysis, leading to apparent elevated Zn concentrations
314 (Figure SI-9 C).

315

316 *Effect of TiO_2 ENMs in wastewater, media and humic acid*

317 Despite the differences in stability, TiO_2 ENMs had no adverse effect on algae growth or
318 photosynthesis in media, humic acid or wastewater, with some experiments even showing slightly
319 increased growth relative to the control (Figure 3). Kulacki and Cardinale also observed increased
320 growth of a range of algal species in the presence of TiO_2 ENMs.⁴⁶ To validate the experimental
321 setup, the experiments were conducted in parallel with positive control herbicide terbutryn, which
322 inhibits photosynthesis with a 50 % effect concentration (EC_{50}) of $7.24 \mu\text{g L}^{-1}$.⁴⁷ Figure SI-11 shows
323 significant growth and photosynthesis inhibition by terbutryn in media, humic acid and wastewater,
324 indicating that the different test matrices were not interfering with the measurement technique.

325 The experiments were conducted at a concentration of 1 mg L^{-1} TiO_2 , which is around 50
326 times higher than detected TiO_2 ENM concentrations in wastewater effluent.¹¹ TiO_2 ENM toxicity
327 towards *P. subcapitata* varies within the literature, with 72 hour growth EC_{50} values ranging from
328 2.5 to 241 mg L^{-1} TiO_2 .^{43, 48-50} The considerable variation may be related to preparation method,
329 extent of TiO_2 aggregation/agglomeration, media type and TiO_2 crystal form (anatase, rutile),

330 though some studies lack proper characterisation of TiO₂ ENMs, making the results difficult to
331 interpret. Further, TiO₂ is known to be photoreactive and may induce formation of reactive oxygen
332 species (ROS) under light conditions.⁵¹ This can be problematic for algal toxicity testing, given that
333 algae require light for growth. However, Miller *et al.* found that TiO₂ ENMs were only toxic to
334 marine phytoplankton under UV light conditions, with most artificial fluorescent lights emitting
335 little UV light.⁵²

336

337 *Effect of ZnO ENMs in wastewater, media and humic acid*

338 In contrast to the TiO₂ ENMs, both types of ZnO ENMs caused growth inhibition in all studied
339 matrices (Figure 4). The effect was similar to that of the positive control ZnCl₂. To compare the
340 effect of ZnO to ZnCl₂, all results for ZnO ENMs were normalised to Zn concentration. Hence 0.1
341 mg_{Zn} L⁻¹ translates to 0.12 mg L⁻¹ for ZnO, which is around 80 times higher than the concentration
342 of ZnO ENMs previously detected in wastewater effluent.²⁸ The presence of organic matter
343 appeared to initially delay growth inhibition of both ZnO ENMs and ZnCl₂ in the humic acid and
344 wastewater experiments, but growth inhibition is similar to media after 72 hours. Photosynthesis
345 inhibition relative to the control was much lower than growth inhibition, indicating that
346 photosynthesis inhibition was not an important toxicity mechanism. Instead, an indirect toxicity
347 mechanism, specifically the release of dissolved Zn, is more likely to explain the observed growth
348 inhibition, given the similarity in effect between ZnO ENMs and ZnCl₂. This is also supported by
349 the ZnO ENM dissolution experiments in humic acid. Dissolved Zn can interfere with the cell
350 membrane of green algae by disrupting calcium uptake.⁵³ A number of studies have attributed ZnO
351 ENM toxicity to *P. subcapitata* to the release of dissolved Zn^{29, 48}, with EC₅₀ values around 0.04 to
352 0.07 mg L⁻¹.

353 There was considerably more variability associated with the results of the effect study for
354 ZnO ENMs compared to TiO₂ ENMs due to the absence of EDTA from the test media. EDTA is
355 often used in algae growth media as it can improve the test performance,⁵⁴ but EDTA is able to
356 complex free metal ions and thus can underestimate toxicity from dissolved Zn. The algae doubling
357 time in the control experiments with and without EDTA were similar for media and humic acid, but
358 algae doubling time was greatly increased in wastewater without EDTA, meaning lower algae
359 counts after 72 hours (Figure SI-12 and Table SI-3). This is most likely due to the presence of trace
360 metals in the secondary treated effluent that were bound by EDTA in the TiO₂ experiments, but
361 were bioavailable in the EDTA free experiments. ICP-MS analysis of wastewater control after
362 microwave digestion indicated that most of the targeted metals (e.g. lead and arsenic) were similar
363 to the experiment blank (2 % HNO₃ in ultrapure water), with the exception of manganese, which
364 was over 10 times higher than the blank.

365

366 **Conclusions**

367 This study aimed to explore the influence of wastewater-derived organic matter and reference
368 organic matter on the behaviour and effect of two common ENMs, TiO₂ and ZnO. The findings are
369 ENM specific. TiO₂ ENMs behaved very differently in wastewater compared to humic acid. While
370 the majority of TiO₂ remained in solution in humic acid after 72 hours (only 25% reduction in
371 concentration), the TiO₂ concentration in the wastewater supernatant was similar to background
372 levels after 24 hours. Additional characterisation confirmed that TiO₂ was not stable in wastewater,
373 with aggregation and/or agglomeration and subsequent sedimentation likely to contribute to the loss
374 from solution. However, as TiO₂ was non-toxic to *P. subcapitata* at the studied concentration, no
375 difference in effect was observed between humic acid and wastewater, despite humic acid
376 stabilising the TiO₂ ENMs.

377 In contrast, both studied ZnO ENM types caused growth inhibition of algae, but this was
378 attributed to the release of dissolved Zn, rather than the nanoparticle form. Both humic acid and
379 wastewater initially suppressed the effect of ZnO ENMs compared to media, but growth inhibition
380 was similar in all matrices after 72 hours. ZnO ENMs appeared to be slightly more stable in humic
381 acid than wastewater, but this did not translate to a significant difference in effect.

382 In conclusion, while organic matter did have an influence on ENM fate, the current study
383 did not observe any difference in effect on algae. However, the altered behaviour in wastewater
384 may have implications for the toxicity of other ENMs and this warrants further investigation. In
385 addition, a limitation of this study is that pristine ENMs were used whereas ENMs that reach
386 WWTPs are likely to be transformed by chemical, physical and biological processes.⁵⁵
387 Nevertheless, this study clearly demonstrates the importance of working with realistic
388 environmental matrices when investigating ecotoxicity of ENMs.

389

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399

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511
512

513 **List of Figures**

514

515 **Figure 1:** ENM percent loss and percent dissolution over time in the algae toxicity tests. A) Ti loss
516 from solution (%) in media, humic acid and wastewater; B) Zn loss from solution (%) in humic
517 acid and wastewater and C) Zn dissolution (%) in humic acid. In B) and C) the closed symbols are
518 NM-110 and the open symbols are NM-112.

519

520 **Figure 2:** Median particle diameter (D_{50}) in media, humic acid and wastewater (with and without
521 algae) for A) TiO_2 , B) ZnO NM-110 and C) ZnO NM-112.

522

523 **Figure 3:** Algae growth inhibition (closed symbols) and photosynthesis inhibition (open symbols)
524 in the presence of $1 \text{ mg L}^{-1} \text{ TiO}_2$ in Talaquil growth media, humic acid and wastewater. Growth and
525 photosynthesis inhibition were calculated using Equations 1 and 2, respectively.

526

527 **Figure 4:** Algae growth inhibition (closed symbols) and photosynthesis inhibition (open symbols)
528 in the presence of $0.1 \text{ mg}_{\text{Zn}} \text{ L}^{-1} \text{ ZnO}$ (NM-110 or NM-112) in A) Talaquil growth media, B) humic
529 acid, and C) wastewater. $0.1 \text{ mg}_{\text{Zn}} \text{ L}^{-1} \text{ ZnCl}_2$ acted as a positive control. Growth and photosynthesis
530 inhibition were calculated using Equations 1 and 2, respectively.

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536 **Supplementary Information: Behaviour of titanium dioxide and zinc**
537 **oxide nanoparticles in the presence of wastewater-derived organic**
538 **matter and implications for algal toxicity**

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541

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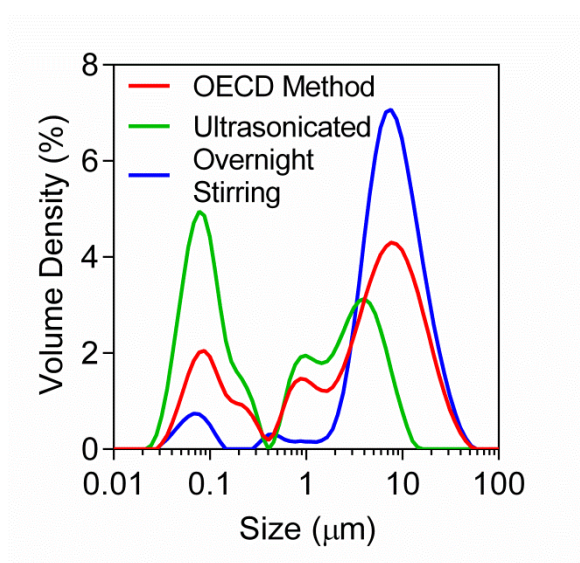
564 SI-1: ENM Preparation Method

565

566 The influence of preparation method on the particle size distribution of TiO₂ was assessed. The
567 preparation methods included stirring overnight, ultrasonication for 1 hour and an adapted OECD
568 method, which involved forming a TiO₂ paste before addition of ultrapure water, then sonication
569 using an ultrasonic probe. Ultrasonication formed the largest amount of small particles, compared to
570 the other two methods.

571

572 Figure SI-1: Influence of preparation method on the volume weighted particle size distribution
573 measured of TiO₂ in ultrapure water (measured by laser diffraction).



574

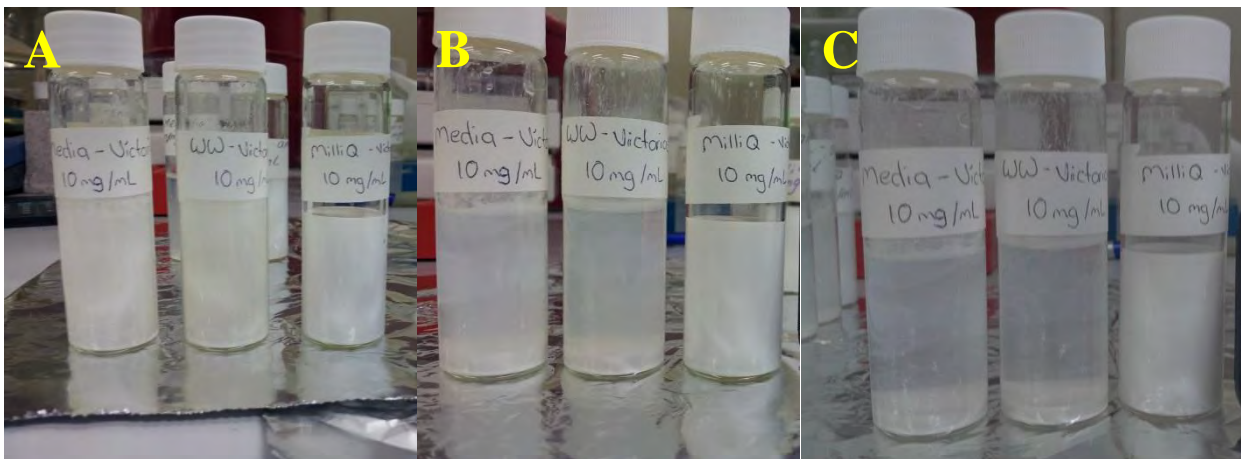
575 SI-2: Visual Stability Assessment

576

577 The influence of media type and preparation method on TiO₂ stability (10 mg/mL concentration)
578 was assessed visually in a time lapse experiment (T=0, 10 and 60 min shown below in Figures SI-2
579 to SI-4). The media types included ultrapure water, Talaquil growth media and wastewater, while
580 the preparation methods were the adapted OECD method (described in Section SI-1), sonication for
581 1 hour and stirring overnight. TiO₂ in ultrapure water appeared to be the most stable, but TiO₂
582 rapidly sedimented out of solution in Talaquil growth media and wastewater. This was most
583 pronounced after sonication and the adapted OECD method. The sonicated sample in ultrapure
584 water appeared to remain in solution for up to 24 hours.

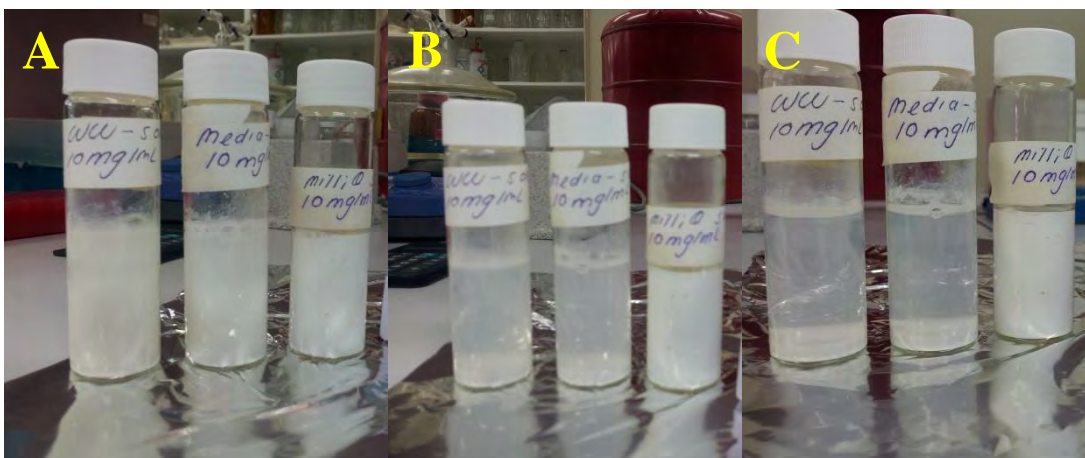
585

586 Figure SI-2: TiO₂ in media, wastewater and ultrapure water after preparation using adapted OECD
587 method at A) T=0, B) T=10 min and C) T=60 min



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589 Figure SI-3: TiO₂ in wastewater, media and ultrapure water after sonication for 1 hour at A) T=0,
590 B) T=10 min and C) T=60 min

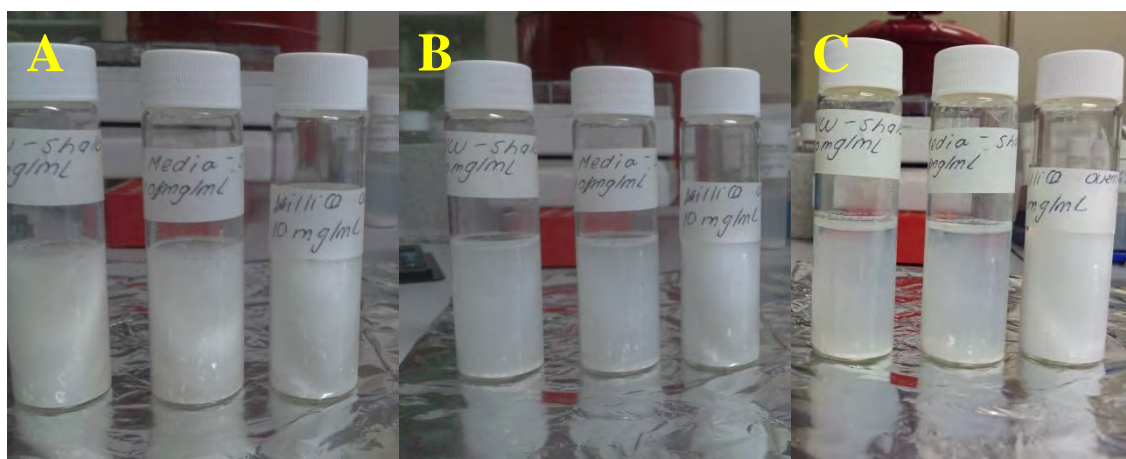


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594 Figure SI-4: TiO_2 in wastewater, media and ultrapure water after shaking overnight for at A) $T=0$,
595 B) $T=10$ min and C) $T=60$ min



596

597 SI-3: Organic Matter Characterisation

598

599 The secondary treated effluent was characterised using liquid chromatography-organic carbon
600 detection (LC-OCD). LC-OCD applies a size exclusion chromatography column to fractionate the
601 sample into different fractions including biopolymers, humic substances, building blocks (degraded
602 humic substances) and low molecular weight neutrals.¹ A HW-50S column (Tosoh, Stuttgart,
603 Germany) with a particle size of 30 μm was used to fractionate the sample. 1000 μL was injected
604 per sample and run for 150 minutes, with a 28 mM phosphate buffer as the mobile phase.

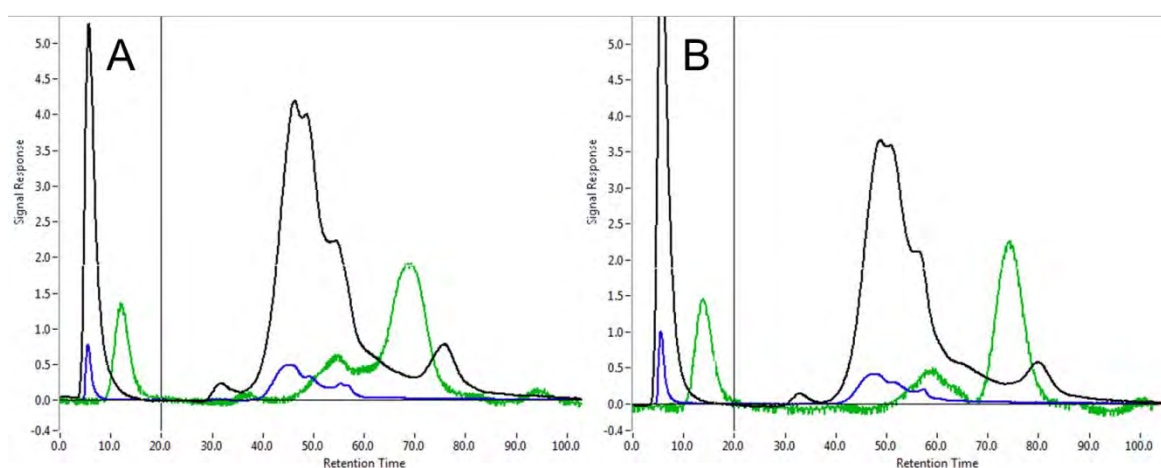
605

606 The total organic carbon concentration after 0.22 μm filtration was 9.25 $\text{mg}_\text{C} \text{L}^{-1}$ in April 2013 and
607 8.3 $\text{mg}_\text{C} \text{L}^{-1}$ in September 2013 after storage for five months, indicating minimal sample
608 degradation over the storage period. LC-OCD was used to investigate structural changes in organic
609 carbon before and after storage, and the black line in Figure SI-1 shows that the organic carbon
610 structure remains similar over time with some degradation of humic substances and a reduction in
611 molecular weight in the September sample.

612

613 Figure SI-5: LC-OCD chromatograms from A) April 2013 and B) September 2013 after storage at
614 pH 2 and 4 $^\circ\text{C}$. The black line shows measurements from the organic carbon detector showing a
615 similar trend in organic carbon structure over time. Ultraviolet detection (blue line) and organic
616 nitrogen detection (green line) also show similar trends over time.

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620 SI-4: Algae growth media

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622 Talaquil growth media contains components including $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NaHCO_3 ,
623 $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, NaNO_3 and 3-(N-morpholino)propanesulfonic acid (MOPS).² It also contains a
624 trace metal mix (Table SI-1) with ethylenediaminetetraacetic acid (EDTA). This helps to improve
625 test performance by increasing precision and reducing false positives.³ However, as it binds free
626 metals, the presence of EDTA will underestimate metal toxicity. Consequently, for the ZnO
627 experiments Talaquil media was prepared without EDTA. The concentration of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and
628 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was also reduced in the EDTA-free stock (shown in italics). The final concentrations
629 of the different trace metal components are shown in Table SI-1.

630

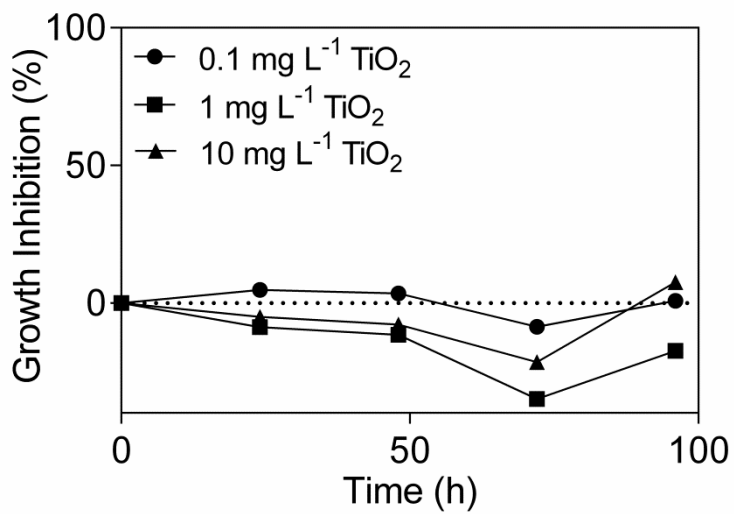
631 Table SI-1: Final concentration in trace metal mix in presence and absence of EDTA.

Components	Standard Talaquil Media Final Concentration (M)	Modified Talaquil Media Final Concentration (M)
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	5.0×10^{-8}	5.0×10^{-8}
H_3BO_3	5.0×10^{-5}	5.0×10^{-5}
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	8.0×10^{-8}	8.0×10^{-8}
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	1.0×10^{-7}	<i>1.0×10^{-10}</i>
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1.0×10^{-6}	1.0×10^{-6}
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1.3×10^{-7}	<i>2.7×10^{-8}</i>
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	9.0×10^{-7}	9.0×10^{-7}
Na_2 EDTA	2.0×10^{-5}	-

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634 Figure SI-6: TiO₂ range finder experiment in Talaquil growth media over 96 hours, with TiO₂
635 concentrations of 0.1, 1 and 10 mg L⁻¹.

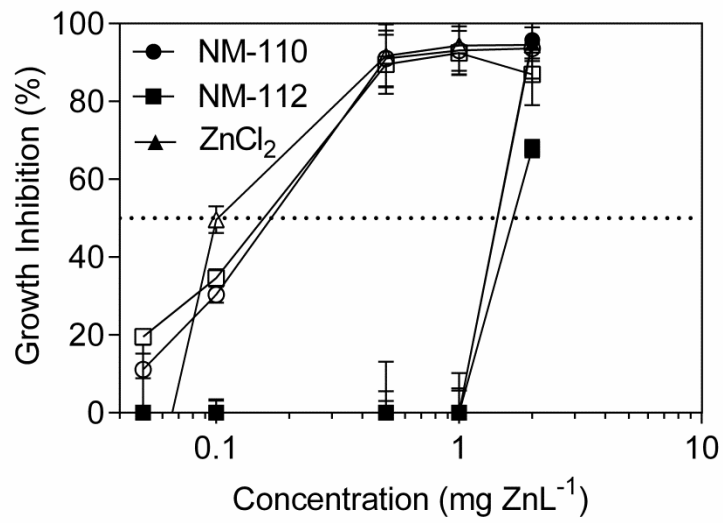


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639 Figure SI-7: ZnO range finder experiment (72 hour time point only) in Talaquil growth media.
640 ZnCl₂ was used as a positive control. Closed symbols are growth media with EDTA and open
641 symbols are growth media without EDTA.



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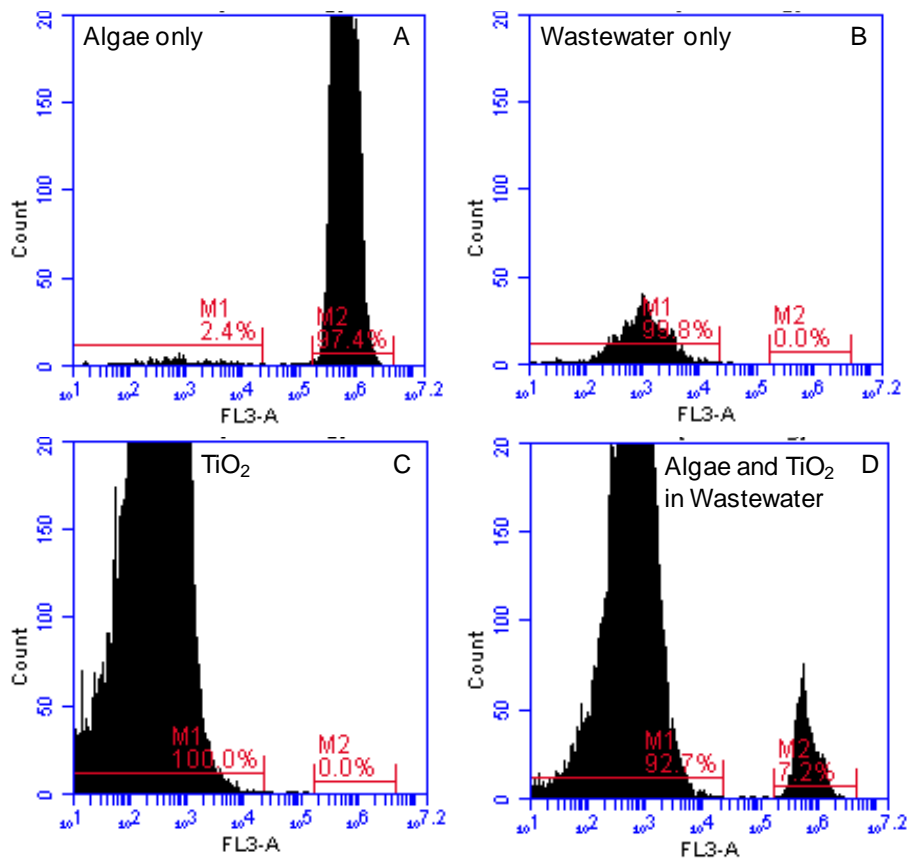
647 SI-5: Flow cytometer validation

648

649 The use of flow cytometry to measure algal cell count was validated by ensuring a distinct algal
650 peak could be measured without interference from organic matter or ENMs in the sample. Algae in
651 media were measured separately and compared to the fluorescence reading of wastewater and TiO₂
652 in wastewater using fluorescence detector FL3, which measures in the wavelength range $\lambda > 670$ nm
653 (Figure SI-4). Gating within the CFlow Plus Analysis software was used to isolate the algal
654 fluorescence peak and obtain a cell count.

655

656 Figure SI-8: Flow cytometer output for A) algae only, B) wastewater only, C) TiO₂ only and D)
657 algae and TiO₂ in wastewater. M2 gating can be used to measure algal count without interference
658 from particles in the wastewater or aggregated TiO₂.



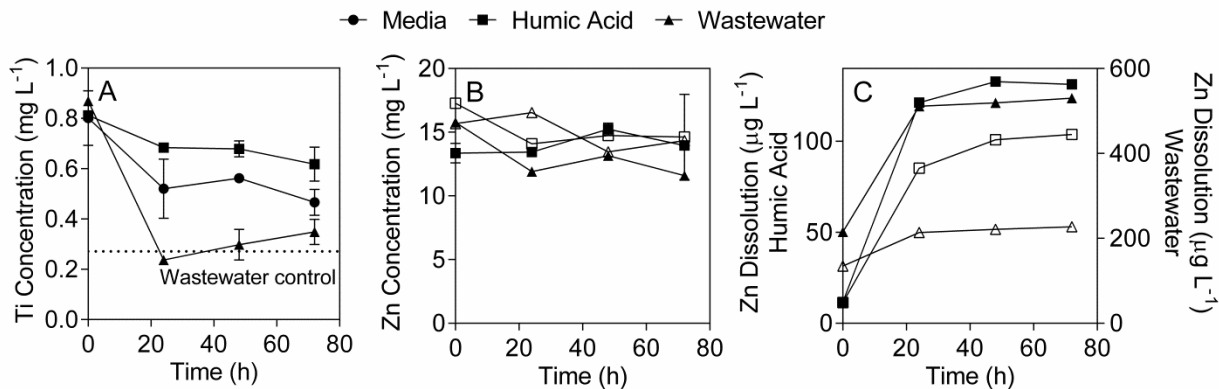
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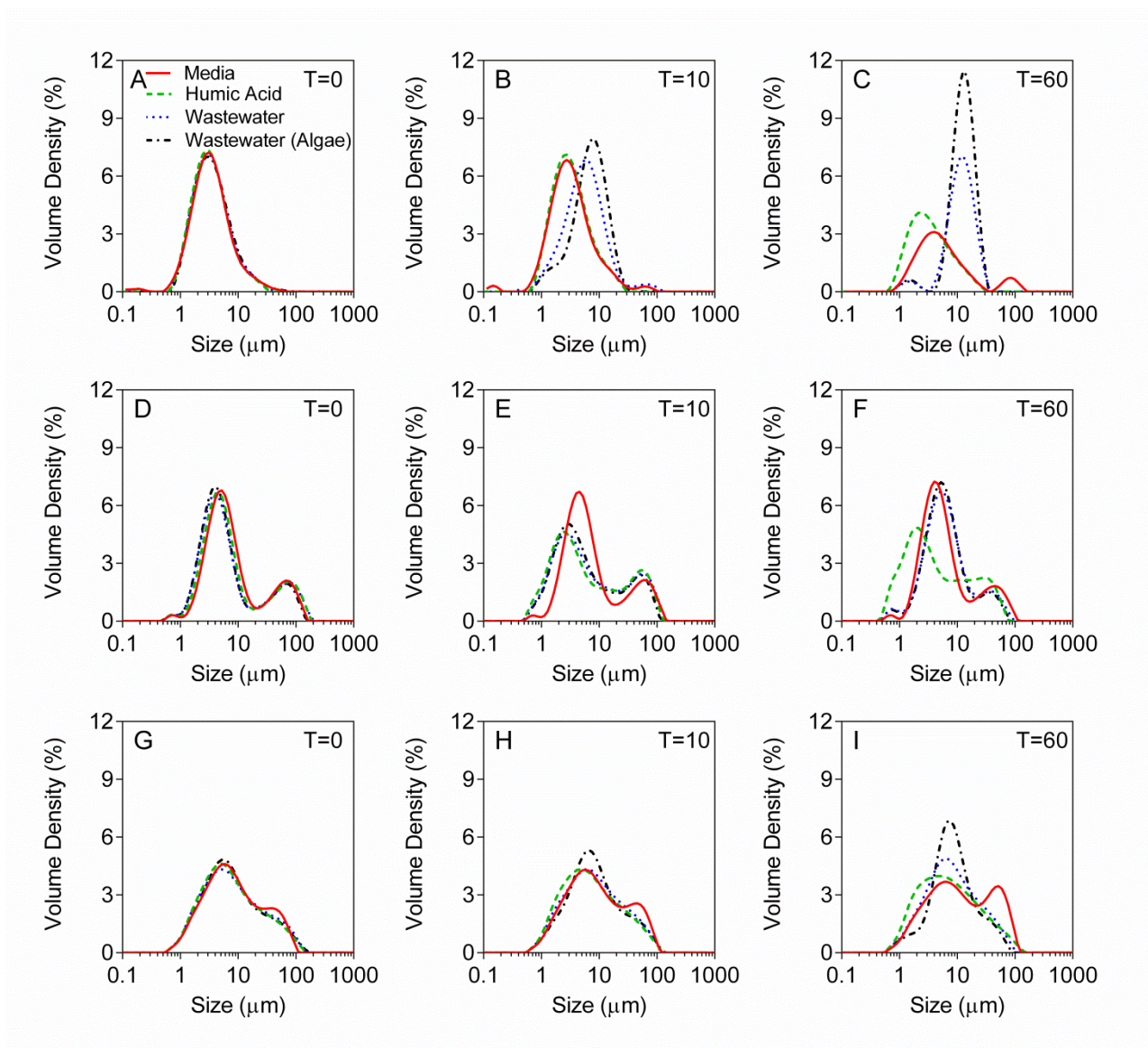
662

663 Figure SI-9: ENM concentration and dissolution over time in the algae toxicity tests. A) Ti loss
 664 from solution (mg L^{-1}) in media, humic acid and wastewater; B) Zn loss from solution (mg L^{-1}) in
 665 humic acid and wastewater and C) Zn dissolution ($\mu\text{g L}^{-1}$) in humic acid and wastewater. In B) and
 666 C) the closed symbols are NM-110 and the open symbols are NM-112.



667

668 Figure SI-10: Time dependence of volume weighted particle size distribution measured by laser
 669 diffraction: A) TiO₂ T=0 min, B) TiO₂ T=10 min, C) TiO₂ T=60 min, D) ZnO NM-110 T=0 min, E)
 670 ZnO NM-110 T=10 min, F) ZnO NM-110 T=60 min, G) ZnO NM-112 T=0 min, H) ZnO NM-112
 671 T=10 min, I) ZnO NM-112 T=60 min.



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682 SI-6: Streaming potential measurements

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684 Streaming potential measurements were carried out on the ENMs. To verify the instrument
685 performance, additional measurements were conducted with a commercial zeta-potential standard
686 sample, polystyrene latex (PSL) suspended in MilliQ grade water. To assess the decrease in
687 streaming potential with time for the samples dispersed in wastewater, a blank sample of
688 wastewater (without ENMs added) was also measured for comparison. The results shown in Table
689 SI-2 indicate that the behaviour of the two ZnO ENM samples is very similar to that of the blank
690 wastewater sample, whereas the TiO₂ ENM sample shows a larger decrease in surface potential
691 during the same time interval.

692

693 Table SI-2: Streaming potential summary for TiO₂ and ZnO ENMs in media, humic acid and
694 wastewater

	Streaming Potential (mV)		Difference (mV)	Associated Standard Deviation (mV)
	T=0	T=10		
PSL Standard in MilliQ	-1201	-1200	1	0.005
<i>Media</i>				
TiO ₂	-1202	-1202	0	0.001
ZnO NM-110	-1202	-1203	-1	0.001
ZnO NM-112	-1202	-1202	0	0.001
<i>Humic Acid</i>				
TiO ₂	-1203	-1202	1	0.001
ZnO NM-110	-1203	-1202	1	0.001
ZnO NM-112	-1202	-1203	-1	0.001
<i>Wastewater</i>				
Wastewater only	-1035	-953	82	12.3
TiO ₂	-1063	-919	144	21.6
ZnO NM-110	-1118	-1070	48	7.2
ZnO NM-112	-1035	-952	83	12.5

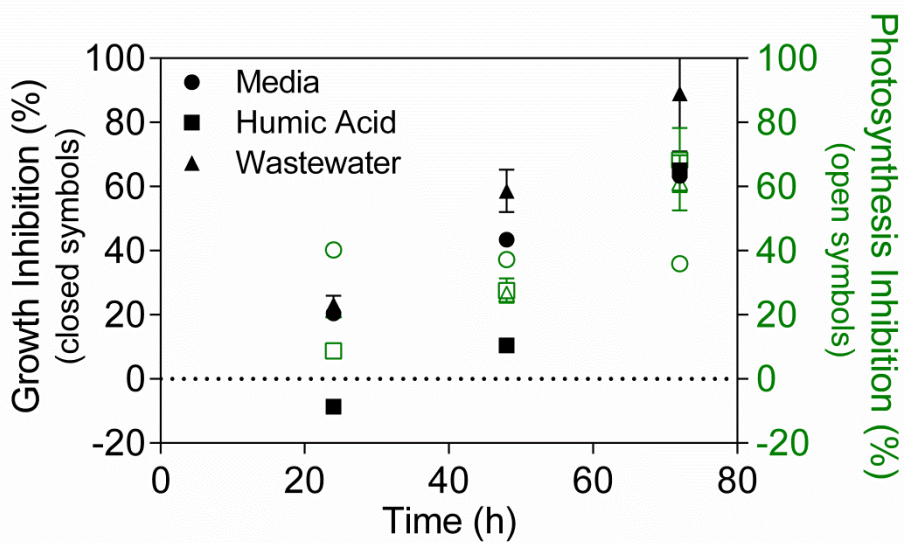
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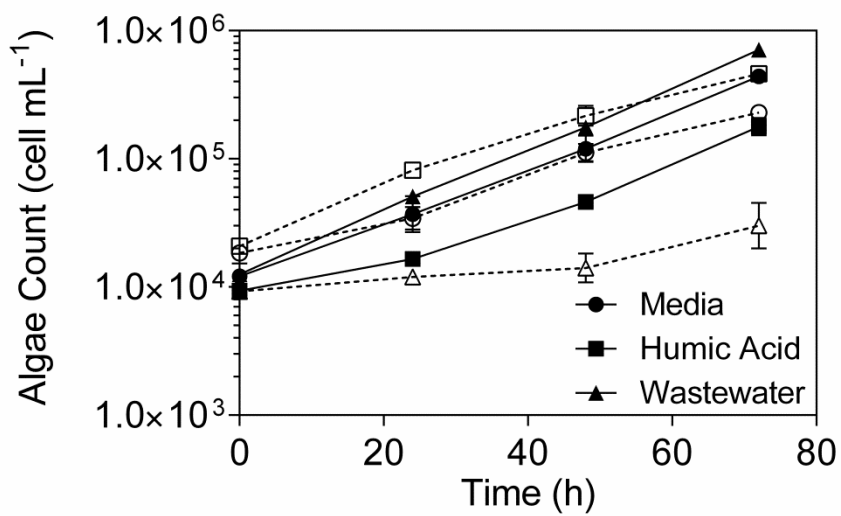
699 Figure SI-11: Algae growth inhibition (closed symbols) and photosynthesis inhibition (open symbols) in the presence of 1.51 $\mu\text{g/L}$ terbutryn (24 hr IPAM EC_{50}) in media, humic acid and
700 symbols) in the presence of 1.51 $\mu\text{g/L}$ terbutryn (24 hr IPAM EC_{50}) in media, humic acid and
701 wastewater.



702

703

704 Figure SI-12: Algae growth over 72 hours in media, humic acid and wastewater control experiments
705 (e.g. without TiO_2 or ZnO). Closed symbols and solid lines are for algae grown with EDTA and
706 open symbols and dashed lines are for algae grown without EDTA.



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712 Table SI-3: Algae doubling time (hours) over each 24 hour period in media, 10 mg_CL⁻¹ humic acid
 713 and wastewater. Without EDTA, the algae doubling time was much longer in wastewater compared
 714 to humic acid and media, leading to a reduced cell count in the wastewater control.

Time	Media		Humic Acid		Wastewater	
	<i>EDTA</i>	<i>No EDTA</i>	<i>EDTA</i>	<i>No EDTA</i>	<i>EDTA</i>	<i>No EDTA</i>
24 hr	14.9	26.7	29.3	12.2	11.2	64.2
48 hr	14.2	14.1	16.2	17.0	13.4	102.9
72 hr	12.8	23.1	12.3	22.0	11.9	21.8

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