Supplementary Information: 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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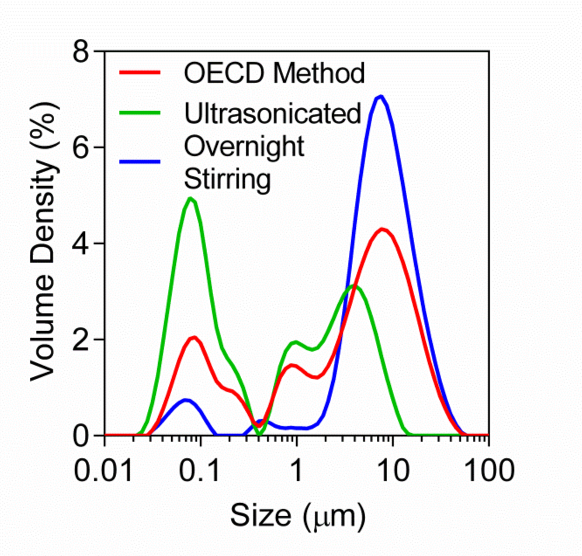
SI-1: ENM Preparation Method

The influence of preparation method on the particle size distribution of TiO_2 was assessed. The preparation methods included stirring overnight, ultrasonication for 1 hour and an adapted OECD method, which involved forming a TiO_2 paste before addition of ultrapure water, then sonication using an ultrasonic probe. Ultrasonication formed the largest amount of small particles, compared to the other two methods.

Figure SI-1: Influence of preparation method on the volume weighted particle size distribution measured of TiO_2 in ultrapure water (measured by laser diffraction).



SI-2: Visual Stability Assessment

The influence of media type and preparation method on TiO_2 stability (10 mg/mL concentration) was assessed visually in a time lapse experiment (T=0, 10 and 60 min shown below in Figures SI-2 to SI-4). The media types included ultrapure water, Talaquil growth media and wastewater, while the preparation methods were the adapted OECD method (described in Section SI-1), sonication for 1 hour and stirring overnight. TiO_2 in ultrapure water appeared to be the most stable, but TiO_2 rapidly sedimented out of solution in Talaquil growth media and wastewater. This was most pronounced after sonication and the adapted OECD method. The sonicated sample in ultrapure water appeared to remain in solution for up to 24 hours.

Figure SI-2: TiO_2 in media, wastewater and ultrapure water after preparation using adapted OECD method at A) T=0, B) T=10 min and C) T=60 min

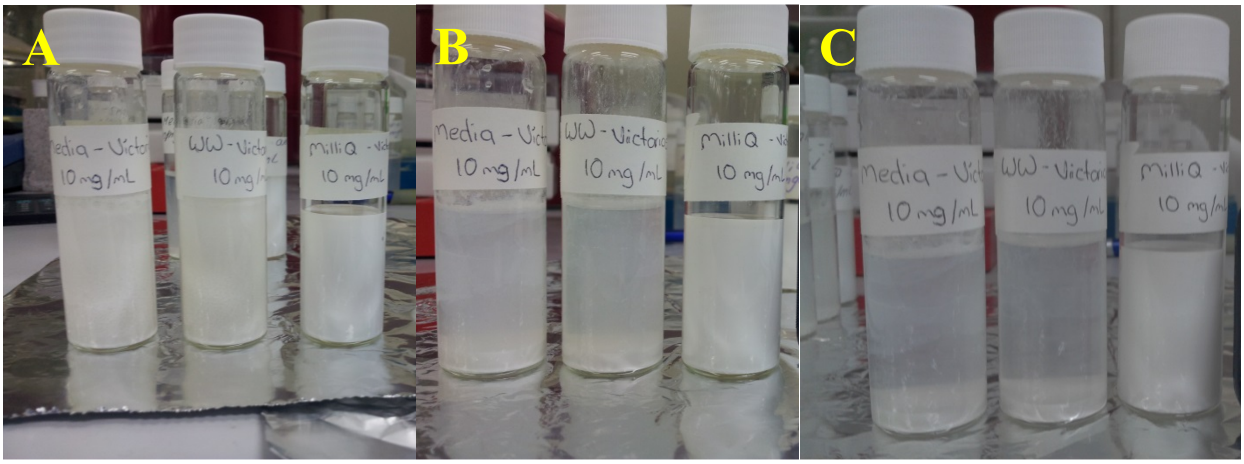


Figure SI-3: TiO_2 in wastewater, media and ultrapure water after sonication for 1 hour at A) T=0, B) T=10 min and C) T=60 min

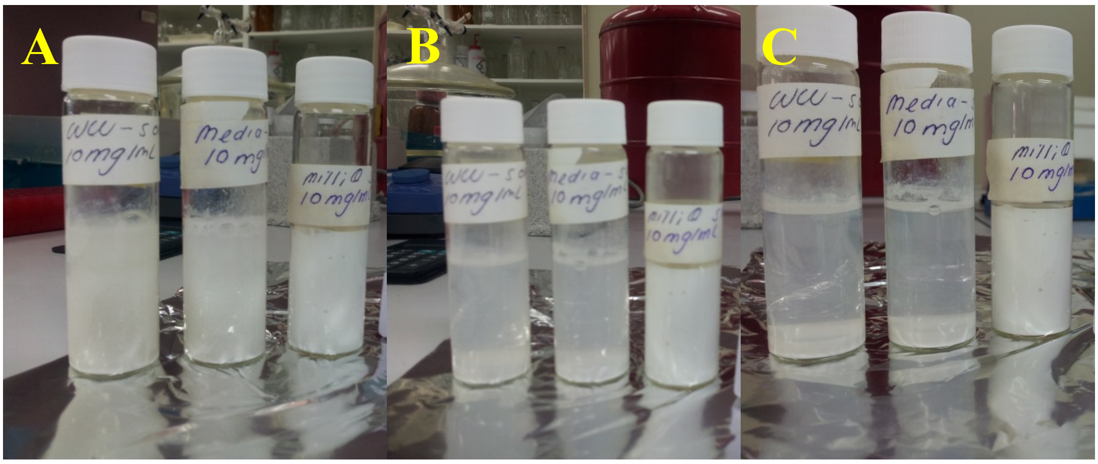
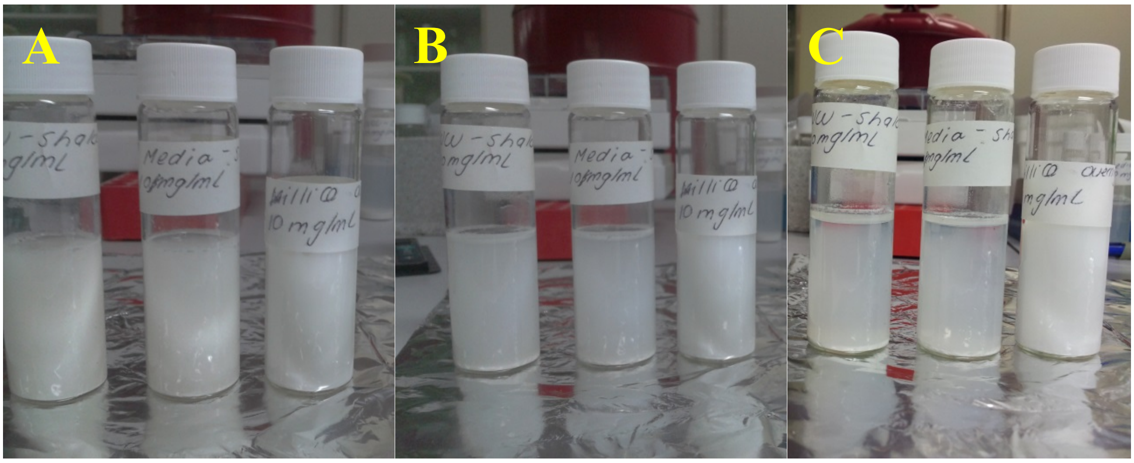


Figure SI-4: TiO_2 in wastewater, media and ultrapure water after shaking overnight for at A) T=0, B) T=10 min and C) T=60 min

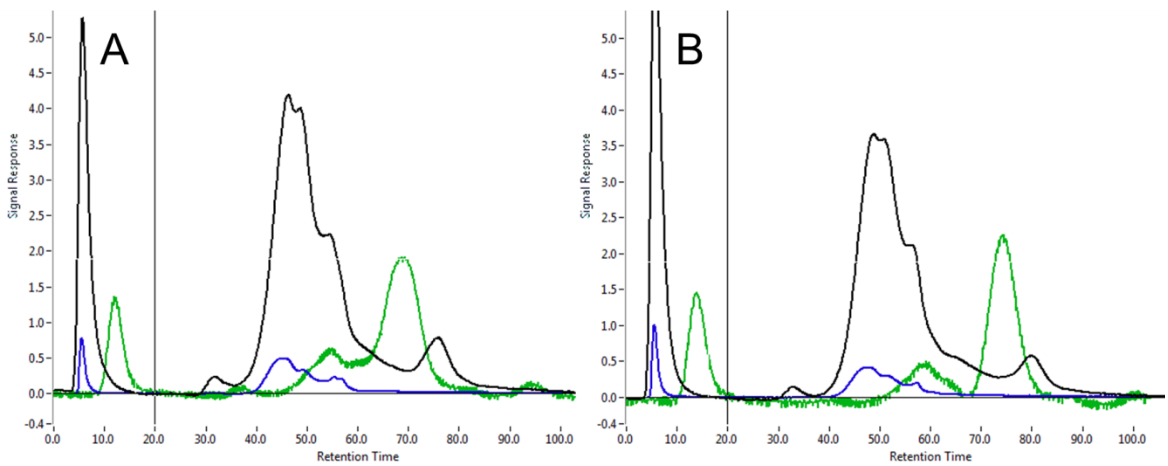


SI-3: Organic Matter Characterisation

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

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

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



SI-4: Algae growth media

Talaquil growth media contains components including $CaCl_2·2H_2O$, $MgSO_4·7H_2O$, $NaHCO_3$, $K_2HPO_4·3H_2O$, $NaNO_3$ and 3-(N-morpholino)propanesulfonic acid (MOPS).[2] It also contains a trace metal mix (Table SI-1) with ethylenediaminetetraacetic acid (EDTA). This helps to improve test performance by increasing precision and reducing false positives.[3] However, as it binds free metals, the presence of EDTA will underestimate metal toxicity. Consequently, for the ZnO experiments Talaquil media was prepared without EDTA. The concentration of $CuSO_4·5H_2O$ and $ZnSO_4·7H_2O$ was also reduced in the EDTA-free stock (shown in italics). The final concentrations of the different trace metal components are shown in Table SI-1.

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)
$CoCl_2·6H_2O$	5.0×10^{-8}	5.0×10^{-8}
H_3BO_3	5.0×10^{-5}	5.0×10^{-5}
$Na_2MoO_4·2H_2O$	8.0×10^{-8}	8.0×10^{-8}
$CuSO_4·5H_2O$	1.0×10^{-7}	*1.0×10^{-10}*
$MnCl_2·4H_2O$	1.0×10^{-6}	1.0×10^{-6}
$ZnSO_4·7H_2O$	1.3×10^{-7}	*2.7×10^{-8}*
$FeCl_3·6H_2O$	9.0×10^{-7}	9.0×10^{-7}
Na_2 EDTA	2.0×10^{-5}	-

Figure SI-6: TiO_2 range finder experiment in Talaquil growth media over 96 hours, with TiO_2 concentrations of 0.1, 1 and 10 mg L^{-1}.

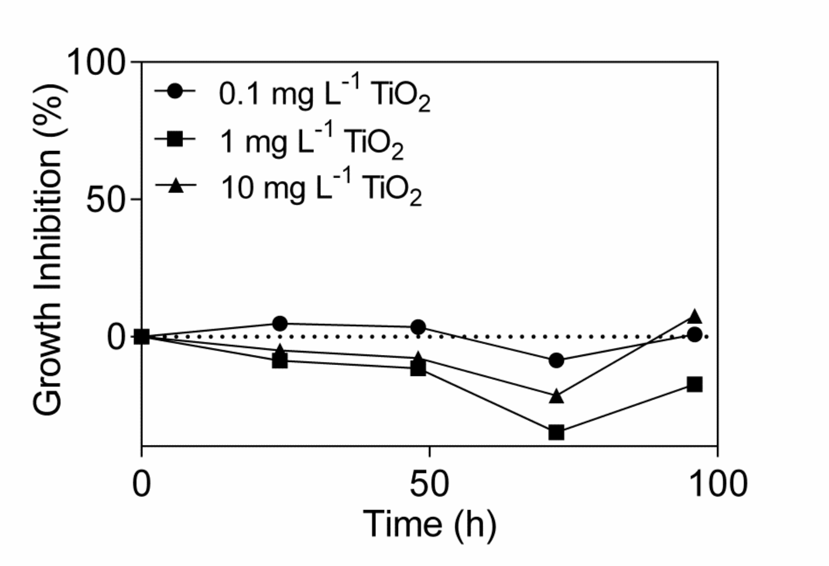
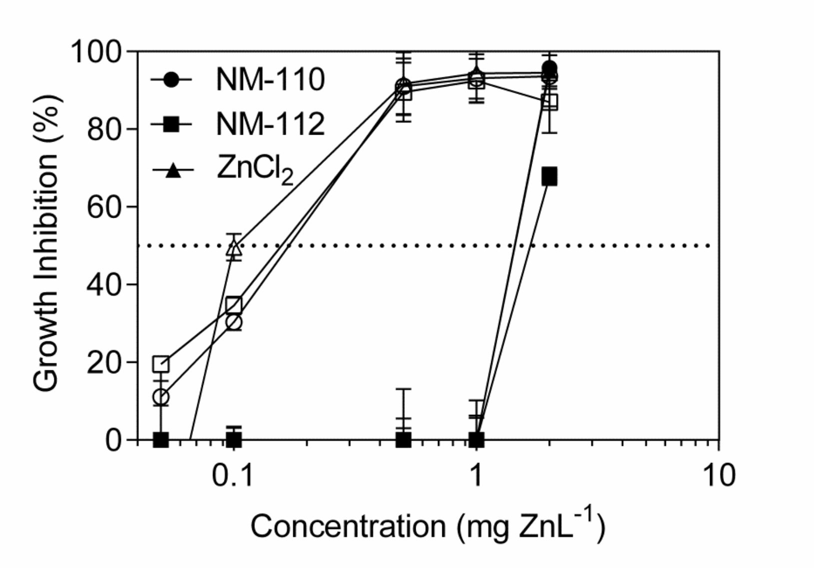


Figure SI-7: ZnO range finder experiment (72 hour time point only) in Talaquil growth media. $ZnCl_2$ was used as a positive control. Closed symbols are growth media with EDTA and open symbols are growth media without EDTA.



SI-5: Flow cytometer validation

The use of flow cytometry to measure algal cell count was validated by ensuring a distinct algal peak could be measured without interference from organic matter or ENMs in the sample. Algae in media were measured separately and compared to the fluorescence reading of wastewater and TiO_2 in wastewater using fluorescence detector FL3, which measures in the wavelength range λ >670 nm (Figure SI-4). Gating within the CFlow Plus Analysis software was used to isolate the algal fluorescence peak and obtain a cell count.

Figure SI-8: Flow cytometer output for A) algae only, B) wastewater only, C) TiO_2 only and D) algae and TiO_2 in wastewater. M2 gating can be used to measure algal count without interference from particles in the wastewater or aggregated TiO_2.

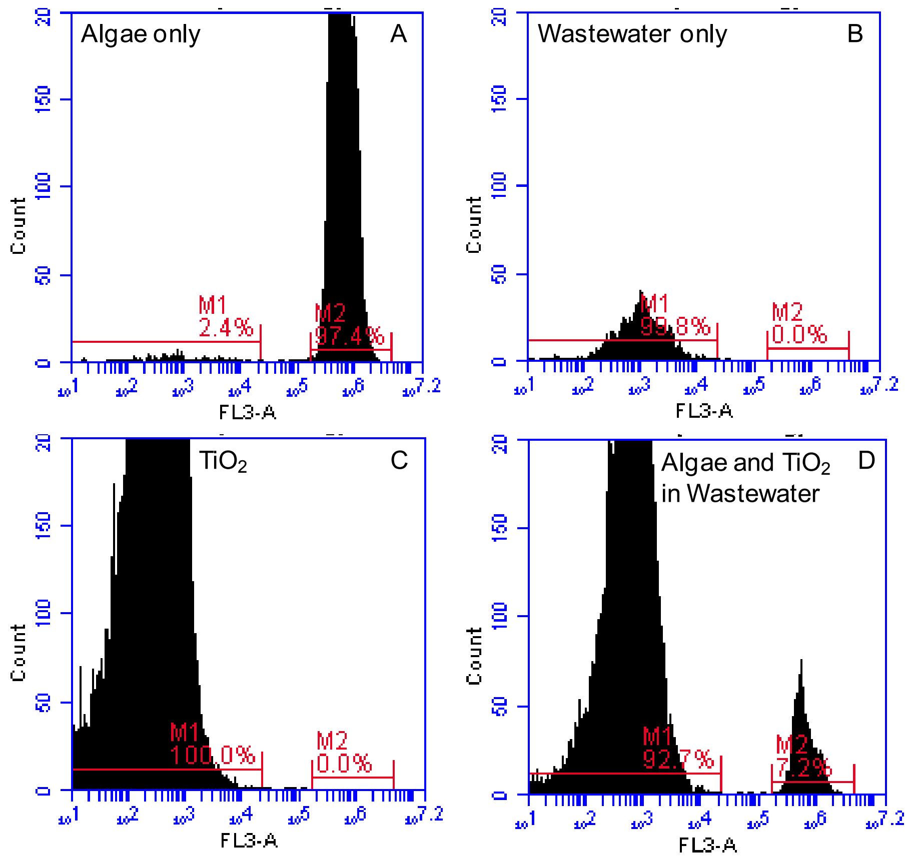


Figure SI-9: ENM concentration and dissolution over time in the algae toxicity tests. A) Ti loss from solution (mg L^{-1}) in media, humic acid and wastewater; B) Zn loss from solution (mg L^{-1}) in humic acid and wastewater and C) Zn dissolution (µg L^{-1}) in humic acid and wastewater. In B) and C)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

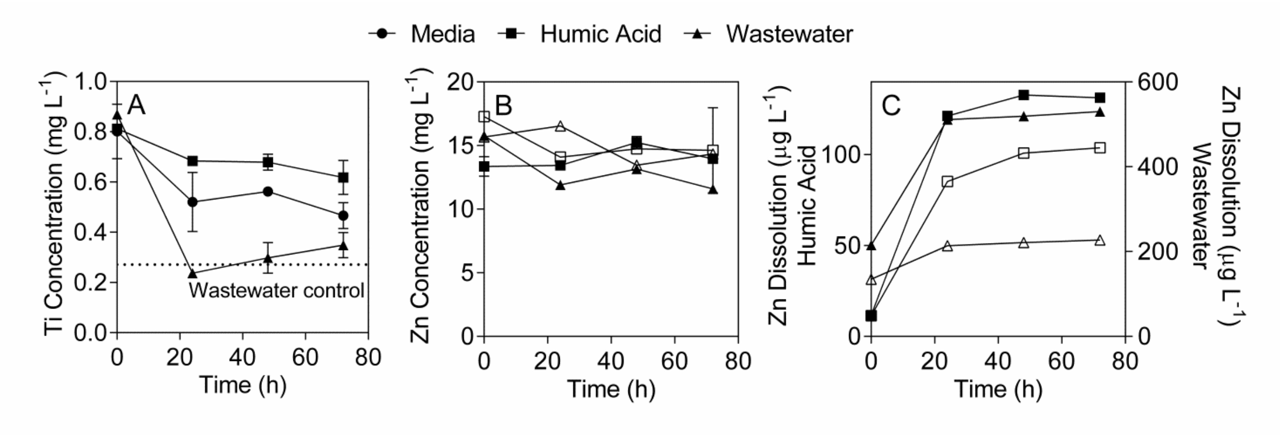
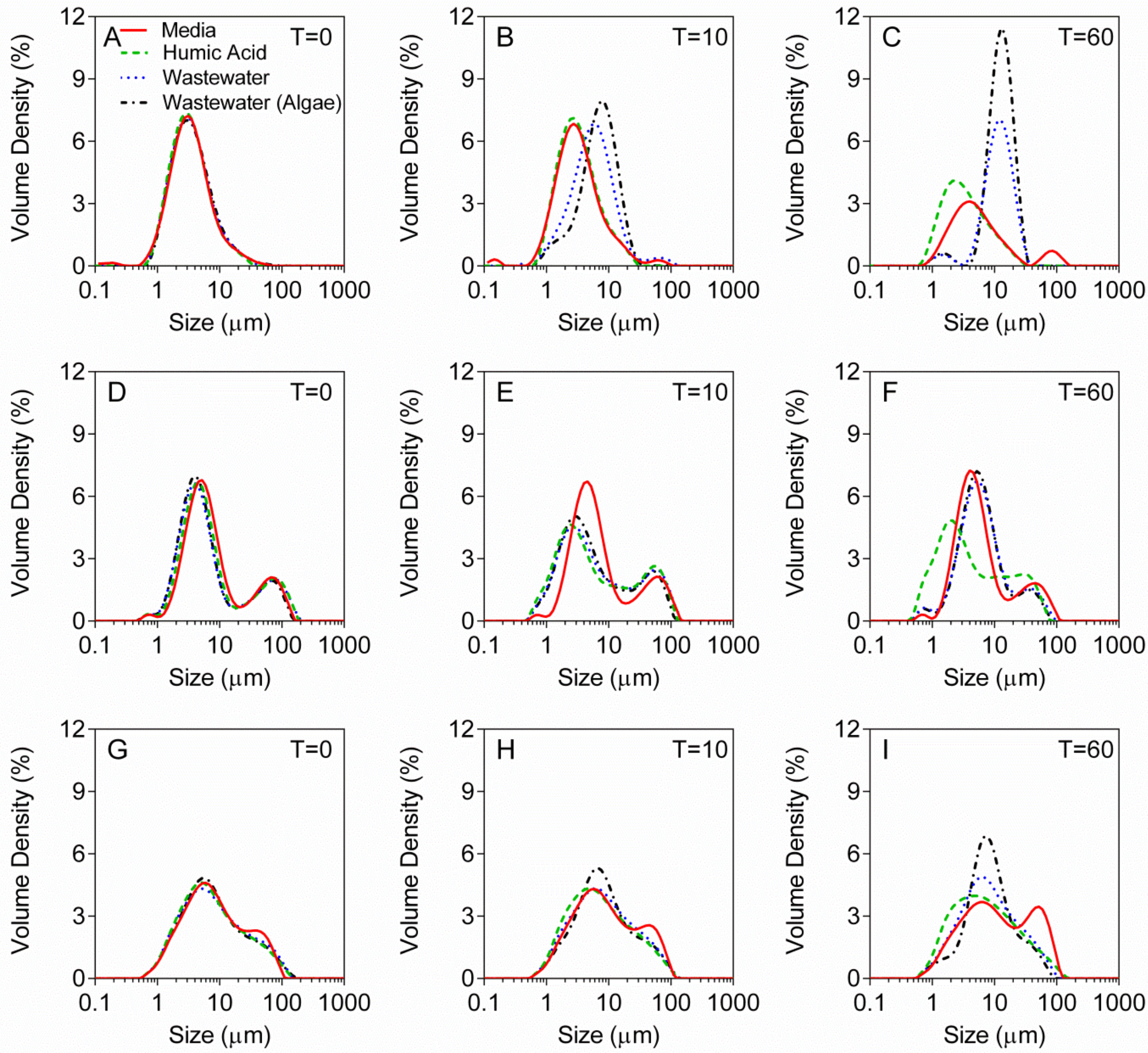


Figure SI-10: Time dependence of volume weighted particle size distribution measured by laser diffraction: A) TiO_2 T=0 min, B) TiO_2 T=10 min, C) TiO_2 T=60 min, D) ZnO NM-110 T=0 min, E) ZnO NM-110 T=10 min, F) ZnO NM-110 T=60 min, G) ZnO NM-112 T=0 min, H) ZnO NM-112 T=10 min, I) ZnO NM-112 T=60 min.



SI-6: Streaming potential measurements

Streaming potential measurements were carried out on the ENMs. To verify the instrument performance, additional measurements were conducted with a commercial zeta-potential standard sample, polystyrene latex (PSL) suspended in MilliQ grade water. To assess the decrease in streaming potential with time for the samples dispersed in wastewater, a blank sample of wastewater (without ENMs added) was also measured for comparison. The results shown in Table SI-2 indicate that the behaviour of the two ZnO ENM samples is very similar to that of the blank wastewater sample, whereas the TiO_2 ENM sample shows a larger decrease in surface potential during the same time interval.

Table SI-2: Streaming potential summary for TiO_2 and ZnO ENMs in media, humic acid and wastewater

	Streaming Potential (mV)		**Difference (mV)**	**Associated Standard Deviation (mV)**
	T=0	T=10		
PSL Standard in MilliQ	-1201	-1200	1	0.005
	Media			
TiO_2	-1202	-1202	0	0.001
ZnO NM-110	-1202	-1203	-1	0.001
ZnO NM-112	-1202	-1202	0	0.001
	Humic Acid			
TiO_2	-1203	-1202	1	0.001
ZnO NM-110	-1203	-1202	1	0.001
ZnO NM-112	-1202	-1203	-1	0.001
	Wastewater			
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

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

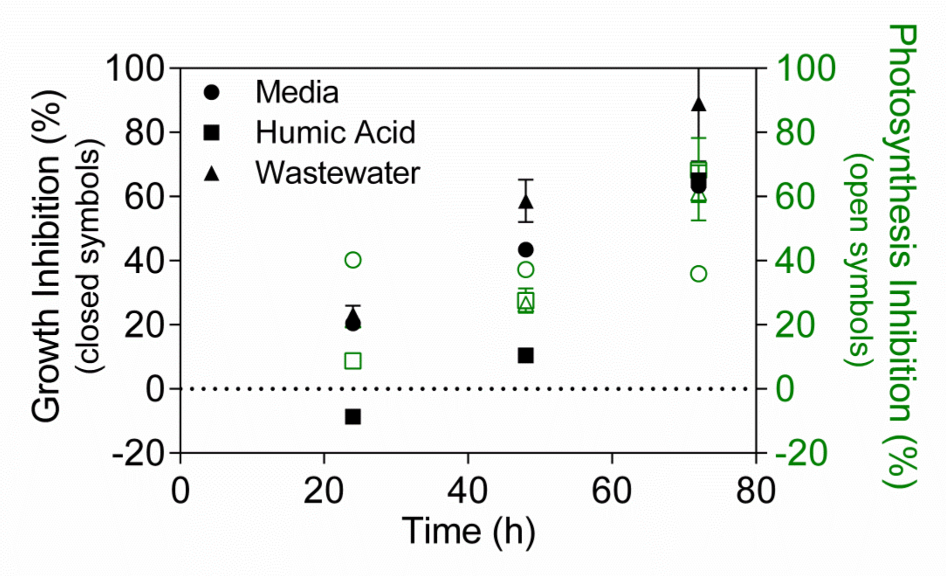


Figure SI-12: Algae growth over 72 hours in media, humic acid and wastewater control experiments (e.g. without TiO_2 or ZnO). Closed symbols and solid lines are for algae grown with EDTA and open symbols and dashed lines are for algae grown without EDTA.

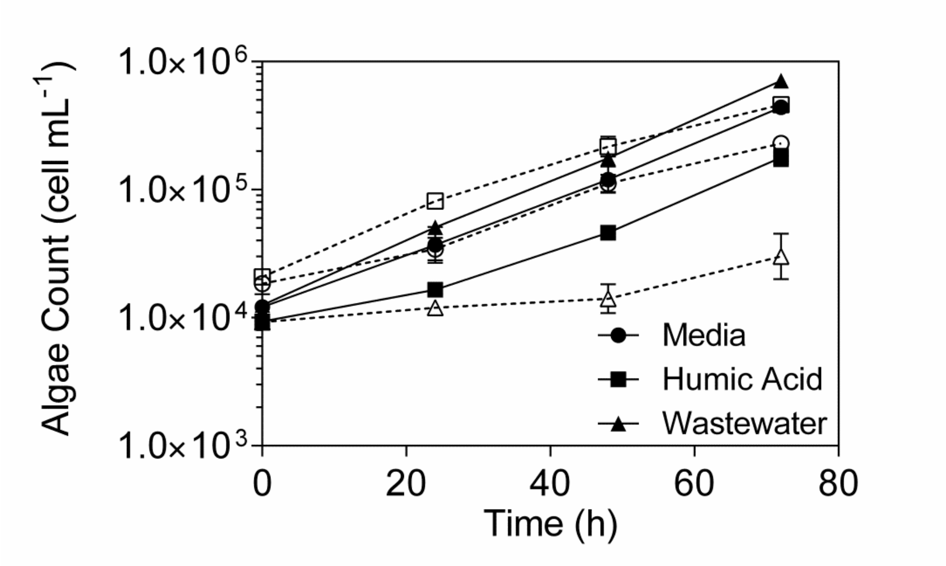


Table SI-3: Algae doubling time (hours) over each 24 hour period in media, 10 $mg_C L^{-1}$ humic acid and wastewater. Without EDTA, the algae doubling time was much longer in wastewater compared to humic acid and media, leading to a reduced cell count in the wastewater control.

Time	Media		Humic Acid		Wastewater	
	EDTA	*No EDTA*	*EDTA*	*No EDTA*	*EDTA*	*No EDTA*
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

References

1. S. A. Huber, A. Balz, M. Abert and W. Pronk, *Water Research*, 2011, 45, 879-885.

2. S. Le Faucheur, R. Behra and L. Sigg, *Environmental Toxicology and Chemistry*, 2005, 24, 1731-1737.

3. U.S. Environmental Protection Agency, *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms (Fourth Edition)*, 2002.