Electronic Supplementary Material (ESI) for Environmental Science: Nano.

SUPPLEMENTARY INFORMATION

The First Comprehensive Safety Study of Magnéli Phase Titanium Suboxides Reveals No Acute Environmental Hazard

Anita Jemec Kokalj[1,*], Sara Novak[1], Iva Talaber[1], Veno Kononenko[1], Lilijana Bizjak Mali[1], Maša Vodovnik[1], Bojana Žegura[2], Tina Eleršek[2], Gabriela Kaličikova[3], Andreja Žgajnar Gotvajn[3], Slavko Kralj[4], Darko Makovec[4], Hana Čaloudova[5], Damjana Drobne[1,*]

[1]*University of Ljubljana, Biotechnical Faculty, Jamnikarjeva 101, 1000 Ljubljana, Slovenia*
[2]*National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia*
[3]*University of Ljubljana, Faculty of Chemistry and Chemical Technology, Večna pot 113, 1000 Ljubljana, Slovenia*
[4]*Institut "Jožef Stefan", Jamova cesta 39, 1000 Ljubljana, Slovenia*
[5]*University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Palackého třída 1946/1,612 42 Brno, Czech republic*

***Corresponding authors:** Anita Jemec Kokalj; anita.jemec@bf.uni-lj.si and Damjana Drobne, Damjana.drobne@bf.uni-lj.si

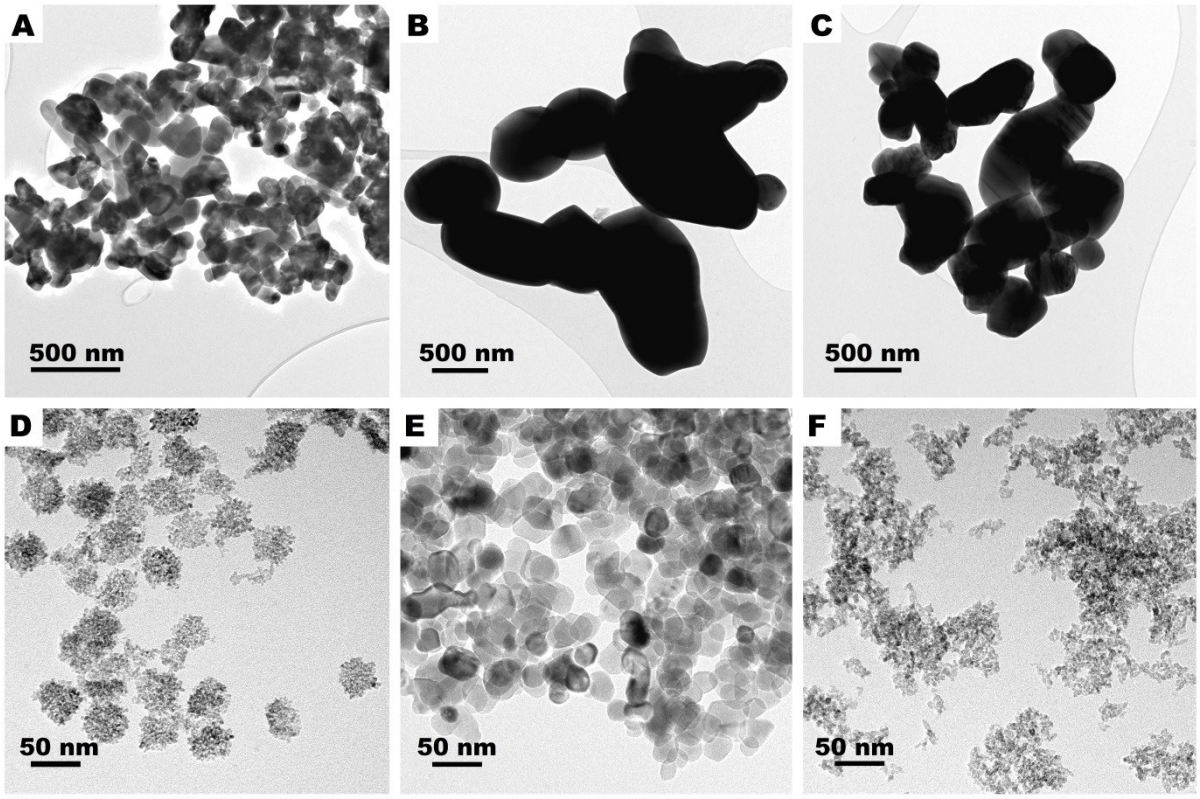

Fig. S1 Transmission electron microscopy images of titanium oxide particles. Micrographs of (A) Magnéli TiO_x-A (B) Magnéli TiO_x-B (C) Magnéli TiO_x-C (D) TiO_2-A (E) TiO_2-B (F) TiO_2-C. Note that the scale bars are different.

TABLE S1: Characteristics of TiO_2 and Magnéli TiOx particles in different test media.

	TiO_2-A	**TiO_2-B**	**TiO_2-C**	**Magnéli TiO_x-A**	**Magnéli TiO_x-B A**	**Magnéli TiO_x-C**
	Medium for *Pseudokirchneriella subcapitata*					
Size (DLS) [nm]	11 and 113	94 and 596	786	82 and 451	824	654
pdi	0.107	0.220	0.603	0.178	0.211	0.045
ζ [mV]	-22.1±3.4	-24.8±1.0	-19.2±2.5	-21.1±1.8	-32.3±2.2	-28.3±1.9
	Medium for *Lemna minor*					
Size (DLS) [nm]	863	1248	903	1369	751	823
pdi	NA	0.252	NA	0.002	0.586	0.67
ζ [mV]	-15.9±1.3	-17.5±0.9	-16.5±0.9	-19.7±0.6	-39.4±1.7	-23.1±0.9
	Medium for *Artemia franciscana*					
Size (DLS) [nm]	863	1434	1248	1138	1369	1192
pdi	NA	0.468	NA	0.1336	0.679	0.216
ζ [mV]	-11.7±2.8	0.0±4.5	15.7±2.0	-1.4±7.7	-1.2±5.6	2.6±6.1
	Medium for *Daphnia magna*					
Size (DLS)	946	1573	1138	1647	68and 1138	1249
pdi	0.608	0.127	0.734	NA	0.087	0.217
ζ [mV]	-13.9±0.7	-4.3±0.6	-2.6±1.0	-5.3±0.9	-27.9±0.9	-17.3±0.8
	Medium for *Tetrahymena thermophila*					
Size (DLS) [nm]	32 and 862	903	653	862	1370	1138
pdi	0.033	NA	NA	0.263	0.160	0.089
ζ [mV]	-19.9±2.0	-21.7±2.2	-18.0±1.9	-15.3±1.5	-13.3±2.3	-15.6±1.7
	Medium for *Danio rerio*					
Size (DLS) [nm]	1370	1982	1138	1249	824	1087
pdi	0.749	0.054	0.754	NA	0.470	NA
ζ [mV]	-14.6±0.6	-2.7±3.5	-10.9±1.8	-2.5±1.8	-15.6±1.8	-9.7±2.0
	Medium for HepG2 cells					
Size (DLS) [nm]	54 and 543	43 and 654	103 and 543	69 and 903	78 and 751	142 and 786
pdi	0.080	0.046	0.156	0.151	0.267	0.202
ζ	-21.1±5.7	-0.5±3.7	-7.9±2.8	-5.9±9.4	-3.7±16.1	-5.7±13.5

[mV]						
	Medium for A549 cells					
Size (DLS) [nm]	130 and 624	136 and 824	68	90 and 946	124 and 1087	49 and 1248
pdi	0.140	0.228	NA	0.219	0.272	0.030
ζ [mV]	-20.9±3.7	-0.5±7.5	-7.5±7.3	-20.8±4.2	2.2±5.9	-23.1±4.6

* NA = data not available by DLS

Chapter S1:
Additional data regarding the methods for toxicity testing

Human cell lines A549 and HepG2

The cytotoxicity of Magnéli TiO_x and TiO_2 particles was evaluated using two different human cell lines (A549 and HepG2) with two different assays measuring different endpoint. A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine and 10 % fetal bovine serum (FBS). HepG2 cells were cultured in Minimum Essential Medium (MEM), supplemented with 2 mM L-glutamine, 1 % non-essential amino acids solution and 10 % FBS. Cells were grown at 37 °C in a humidified atmosphere with 5 % CO_2 and were routinely passaged twice a week.

Metabolic activity of exposed cells was measured by **resazurin assa**y. Cells were seeded in black 96-well plates (7000 A549 cells or 8000 HepG2 cells per well, respectively). After 24 h incubation at controlled atmosphere, allowing cells to attached, cells were treated with different concentrations (1, 10, 100 mg/L) of titanium oxide particles prepared in cell culture medium. After 24 h treatment, 25 µg/mL resazurin was added to each well for 3 hours and incubated at 37 °C. Fluorescence intensity of formed resorufin was measured (560/590 nm ex/em) using spectrofluorimeter (BioTek, Cytation 3, Bad Friedrichshall, Germany). For each

treatment condition 3 independent experimental repeats of 6 replicates were performed.

Coomassie Blue assay was used to measure amount of cellular proteins that is proportional to the cell number. Cells were seeded in transparent 96-well plates (7000 A549 cells or 8000 HepG2 cells per well, respectively). After 24 h incubation at controlled atmosphere, allowing cells to attached, cells were treated with different concentrations (1, 10, 100 mg/L) of titanium oxide particles prepared in cell culture medium. After 24 h treatment, cells were rinsed with phosphate-buffered saline (PBS) and stained by Coomassie Blue solution (0.05 % Coomassie Brilliant Blue G250 in 30 % methanol, 10 % acetic acid, 60 % MilliQ water) for 40 min at room temperature. After additional rinsing with PBS, 0.1 M NaOH was added to solubilize the dye. The absorbance of the day was measured at 630 nm using a microplate reader (BioTek, Cytation 3, Bad Friedrichshall, Germany). For each treatment condition 3 independent experimental repeats of 6 replicates were performed.

Toxicity test with algae *Pseudokirchneriella subcapitata*

The OECD 201 - Algal growth inhibition test guidelines (OECD, 2011) were followed, using the *P. subcapitata* (SAG 61.81) as a test organism. All assays were run in three replicates with initial algal cell count of 10000 cells/ml. Test cultures were incubated in 2 mL portions in multiwell plates (Nunclon, Germany) on the orbital shaker (80 rpm) at the temperature of 22 ± 2 °C and constant light exposure (light intensity of 80-120 µmol photons m^{-2} s^{-1}) in the algal growth chamber (LTH, Slovenia). Algal growth and fluorescence spectra in 50 µl from every well were assessed at 24, 48 and 72 h with a flow cytometer (MacsQuant, MiltenyBiotech, Germany). Algal cultures were inspected also under light microscope (Nicon Eclipse TE300, Japan).

The final test result was growth inhibition, expressed as a logarithmic increase in cell count (average specific growth rate) within 72 h of exposure to selected materials.