Supporting Information

Ecotoxicological effects of transformed silver and titanium dioxide nanoparticles in the effluent from a lab-scale wastewater treatment system

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Number of tables: 3

MATERIALS AND METHODS

Synthetic wastewater-influent

The synthetic wastewater was prepared to imitate Scandinavian soft wastewater and was therefore based on a previous study[1] with modifications. It consisted of 127.5 mg/L $CaCl_2$, 210 mg/L $NaHCO_3$, 200 mg/L NaCl, 100 mg/L NH_4Cl, 50 mg/L K_2HPO_4, 300 mg/L powder milk (Viking Melk, Nestle), 60 mg/L potato starch (Potetmel, Hoff), 5.5 mg/L $MnCl_2 \cdot 4H_2O$, 0.68 mg/L $ZnCl_2$, 1.2 mg/L $CoCl_2 \cdot 6H_2O$, 1.2 mg/L $NiCl_2 \cdot 6H_2O$, 2 mg/L $FeCl_2 \cdot 4H_2O$, 15.47 mg/L Na_2-EDTA in tap water. It should be noted that sulfur compounds may be present in the artificial wastewater due to the addition of powder milk. The synthetic wastewater had a pH of 7.7, alkalinity of 3.94, total nitrogen of 33.7 mg N/L, total organic carbon of 124 mg C/L and a COD of 450 mg/L. The synthetic wastewater was prepared every 2-3 days (23 L per day).

Lab-scale wastewater treatment plant.

The lab-scale WWTP was a pre-denitrifying activated sludge treatment system comprising of a 6.5 L non-aerated denitrifying reactor, an 8 L aerated nitrifying reactor with automatic temperature (20°C) and pH (7.2) control and a 5.1 L settler (Figure S1). Prior to the main test, the system was continuously fed (hydraulic retention time of approximately 15 h) synthetic wastewater without any NPs added for a period of 10 weeks to adapt the activated sludge to the synthetic medium and to wash out any NPs transferred to the system together with the initial sludge. The influent flow rate was 16.3 ml/min. The sludge and water return from the settler back to the denitrifying reactor was 2.7 times the influent flow rate (43.8 ml/min). Sludge was continuously removed from the denitrifying reactor (~1.6 L/day) to maintain a solids retention time (SRT) of approximately 15 days. During the adaptation period effluent

samples were collected weekly and served as "background controls". After the adaptation period the synthetic medium was dosed with the aforementioned Ag and TiO_2 NP suspensions to provide a continuous supply of 10 µg/L Ag NPs and 100 µg/L TiO_2 NPs to the denitrifying reactor. The synthetic wastewater containing Ag and TiO_2 NPs was prepared freshly every 2-3 days. This dosing to the system was continued for a period of 5 weeks. The Ag and TiO_2 NPs were added to the synthetic wastewater under rapid stirring, and the medium was maintained under stirring conditions throughout the test period (300 rpm). Effluent samples were collected weekly and used to evaluate the impact of NP transformation through application to the battery of bioassays. The dissolved oxygen (DO) and total suspended solids (TSS) were measured daily in both reactors and in effluent, while ammonium (NH_4^+, HACH Method LCK 303), nitrate (NO_3^-, HACH Method LCK 339), nitrite (NO_2^-, according to the HACH method 8507) and the chemical oxygen demand (COD, HACH Method LCK 314) concentrations were measured weekly throughout the test period until the last three days when sampling was conducted daily. TSS concentration was measured according to the Norwegian standard for the determination of suspended solids method by filtration through glass fibre filters (NS-EN 872:2005)[2]. DO concentrations were maintained at >4 mg O/L in the nitrifying reactor. The COD and total inorganic N removal was 81±8 % and 71±16 %, respectively (% removal= $(C_{infl}-C_{effl})/C_{infl})\cdot 100$).

Ag and TiO_2 NP characterization (STEM/EDS, sp-ICP-MS).

Approximately 1 µl of the Ag or TiO_2 NP stock dispersion was deposited onto a copper grid (holey carbon 400 mesh, Agar Scientific) and allowed to dry through evaporation. For preparation of the effluent and sludge samples two different methods were used. In the first method, 12 ml of sample was placed in a 3KDa cut-off membrane and centrifuged at 5000 g

for 1hr. The resulting 0.5 ml concentrate was diluted 10 times and then a droplet of ~1 µl was deposited on a copper grid. In the second method, grids were placed on the top surface of a 0.7 µm glass fiber filter (glass microfiber GF/F, Whatman, GE Healthcare Life Sciences) on a filtration unit, 10 ml of effluent was then passed through the system followed by a washing step with 2 ml of MilliQ water. STEM was carried out at 300 kV with an FEI Titan G2 60-300 instrument equipped with a DCOR probe Cs-aberration corrector and a Super-X Bruker energy dispersive spectrometer with 4 Si drift detectors. High-angle annular dark field (HAADF) imaging maximized the contrast of heavy-element NPs. A probe current of 100-300 pA, a probe convergence of 22 or 30 mrad and a collection angle of 76-200 mrad were employed for imaging while elemental point analysis and mapping were performed with energy-dispersive X-ray spectroscopy (EDS).

Single particle ICP-MS (sp-ICP-MS) characterization (particle concentration and size) was performed using an Agilent 7700 Q-ICP-MS equipped with a SETAC ASX- 520 autosampler (housed in a laminar flow enclosure to minimize airborne contamination). The ICP-MS instrument is housed in a class 1000 filtered air laboratory. The peristaltic pump sample uptake rate was 0.32 ml/min and the dwell time was set to 3 ms (monitoring ^{107}Ag, ^{197}Au or ^{x}Ti). Data acquisition was done using the Agilent Mass Hunter software in the time resolved analysis (TRA) mode with a 60s acquisition time. We used 30 nm (RM8012) and 60 nm (RM8013) citrate stabilized Au NP reference materials from NIST at a concentration of 50 ng/L (in MQ water) to determine the nebulization efficiency[3]. Data were exported as csv files and processed by the RIKILT calculation tool in Microsoft Excel. Ionic standards of the respective elements were used for calibration. The detection threshold for differentiating NPs from the background signal was determined as the mean of each dataset plus three times the standard deviation (+ 3 x SD).

Ag and TiO_2 fractionation (filtration, ultrafiltration and ICP-MS)

A 10 ml aliquot of each fraction was placed in acid-washed 15 ml centrifuge falcon tubes while the 3kDa cut-off membrane filtrate was kept in the amicon tube. In each sample, 1% 7M HNO_3 was added (Suprapur®, Merck Millipore) and kept at 4°C until ICP-MS analysis (ICP-QQQ with an ASX-520 Autosampler, Agilent 8800, Agilent Technologies, USA). In the filter samples, 5 ml of 65% HNO_3 was added and left over night at room temperature. Then 15 ml MilliQ water and 1 ml 47–51% hydrofluoric acid (HF) were added and the samples placed in an ultrasonic bath for 2 h at 80 °C to dissolve the filters and any TiO_2 completely. Liquid samples with visible particles were also dissolved with HNO_3 and HF (2 ml sample + 1 ml HNO_3 and 0.1 ml HF) and ultrasonicated for 2 h at 80 °C. The other liquid samples were vortexed before analysis. Due to limitations of the instrument regarding HF-concentration, all dissolved samples were diluted in MilliQ water prior to analysis (highest HF concentration 0.2%). For all samples, ^{115}In was added as an internal standard and quantified against standards purchased from Inorganic Ventures.

Mass balance calculation

The mass balance between total amounts of Ag and TiO_2 NPs having entered the system, total amounts having left the system and the amounts in the system at a given time is given below:

$$Mass\ balance = \sum_{n=0}^{35} M_{entered,n} - \sum_{n=0}^{35} M_{left,n} - \sum_{n=0}^{35} M_{system,n}$$

Accumulated amount having entered the system:

$$\sum_{n=0}^{35} M_{entered,n} = \sum_{n=1}^{35} \bar{C}_{inf} \cdot Q_{inf,n} \cdot (t_n - t_{n-1})$$

- $\bar{C}_{inf}$ = average influent concentration in influent (µg/L)
- $Q_{inf,n}$ = Influent flow rate at time n (L/h)
- t_n = duration at time n after the start of exposure (h)
- $t_n - t_{n-1}$ = duration since last sample measurement (h)
- Assumption: Constant concentration in influent based on the average concentrations of nine (Ag) and seven (Ti) measurements. Two Ti measurements outliers

Accumulated amount having left the system:

$$\sum_{n=0}^{35} M_{left,n} = \sum_{n=0}^{35} (C_{eff,n} \cdot Q_{eff,n} + C_{ES,n} \cdot Q_{ES,n}) \cdot (t_n - t_{n-1})$$

- $C_{eff,n}$ = effluent concentration at time n (µg/L)
- $Q_{eff,n}$ = effluent flow rate at time n (L/h)
- $C_{ES,n}$ = concentration in extracted sludge (µg/L)
- $Q_{ES,n}$ = extraction rate (L/h)
- Assumption: Concentration in extracted sludge same as in DNR

Amount in the system:

$$\sum_{n=0}^{35} M_{system,n} = C_{NR,n} \cdot V_{NR} + C_{DNR,n} \cdot V_{DNR} + C_{S,n} \cdot V_S$$

- $C_{NR,n}$ = concentration in nitrifying reactor (µg/L)
- V_{NR} = volume of nitrifying reactor (8.0 L)
- $C_{DNR,n}$ = concentration in denitrifying reactor (µg/L)
- V_{DNR} = volume of denitrifying reactor (6.5 L)
- $C_{S,n}$ = concentration in settler (µg/L)
- V_S = volume of settler (5.1 L)
- Assumption: Average concentration in settler same as in nitrifying reactor

According to mass balance calculations, after 5 weeks of operation of the system ~15% (1.5 mg) of Ag leaving the system was associated with effluent, 67% (6.6 mg) with the excess sludge while 16% (1.6 mg) remained in the system. For Ti, 13% (5 mg) left the system with the effluent, 70.7% (27.5 mg) with the excess sludge and 15.8% (6.6 mg) remained in the system. This leads to a mass closure of 98.3% and 99.5% for Ag and TiO_2, respectively. The TiO_2 NP concentration measured in the influent can be underestimated due to settling. Therefore, measured concentrations of freshly prepared influent media containing Ag and TiO_2 NPs were used for the mass balance calculations. The measured influent concentrations for Ag and Ti were 11.1 ± 0.67 µg/L and 44 ± 11.6 µg Ti/L.

Quantitative real time PCR (qPCR).

The qPCR was performed as previously described[4]. Briefly, reverse transcription (RT) of total RNA (0.25-1 μg) was performed using Quanta qScript™ cDNA synthesis kit (Quanta Biosences Inc.,Gaithersburg, USA) according to the manufacturer's guidelines. The PCR was performed on a CFX-384 thermalcycler (Bio-Rad laboratories Inc., USA) and primer optimization was performed using 5-fold dilutions (3.125-50 ng/reaction) with pooled template cDNA. Primers were selected according to previous studies (Table S3) and purchased from Eurofins (Ebersberg, Germany). SYBR®GreenSupermix (Quanta Biosences Inc., Gaithersburg, USA) was used in the reaction where 10 ng template/reaction were used in the final mastermix reaction (20 μl/reaction) in technical duplicates. Thermal cycling conditions were as follows: Cycle 1 95°C (3 min), Cycles 2-40 95°C (20 s), followed by the specific primer annealing temperature (20 s) and 72°C (20 s). Relative expression was calculated according to the Pfaffl method[5] and the β actin (act-b) gene was used as the reference gene for normalization.

TABLES

Table S1. Characterization of Ag and TiO_2 NP average particles size in MilliQ, synthetic wastewater and effluents collected during weeks 1-5 of dosing of the system, determined using DLS and sp-ICP-MS analysis.

	DLS		sp-ICP-MS	
Sample	Ag particles (nm)	TiO_2 particles (nm)	Ag particles (nm)	TiO_2 particles (nm)
MilliQ	58.8 ± 0.19	640.7 ± 9.2	26.5 ± 0.7	278 ± 15.5
Synthetic wastewater	60.7 ± 0.18	969 ± 19	ND	ND
Seawater	59.3 ± 0.76	1375 ± 76.7	ND	ND
S. pseudocostatum dilution water	59.5 ± 0.18	1369 ± 99.2	ND	ND
R. subcapitata dilution water	57.3 ± 0.17	650 ± 6.4	ND	ND
D. magna EPA media	58 ± 0.04	1274 ± 88.7	ND	ND
RTgill-W1 L15/ex media	58.7 ± 0.2	1287 ± 81	ND	ND
Effluent Wk1	ND	ND	20.5 ± 0.7	119.0
Effluent Wk2	ND	ND	31.6	110.9
Effluent Wk3	ND	ND	27.3 ± 1.6	114.1
Effluent Wk4	ND	ND	24.8	NM
Effluent Wk5	ND	ND	21.2 ± 1.8	124.8
Dissolution% in SWW	2.85	0.5		

ND: not determined

Table S2. Overview of effluent characteristics collected during 5 weeks of dosing of the system with 10 µg/L Ag NPs and 100 µg/L TiO_2 NPs.

Sample	TSS (mg/L)	NH_4-N (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	CODsol	Conductivity (mS/cm)	pH	DO
Bckgr effl	14.3					1.2	7.45	
Week 1	15.7	0.31	10.3	0.77	32.3	1.37	7.52	6.25
Week 2	83	3.33	4.72	0.89	19.6	1.08	7.20	6.70
Week 3	10.85	0.19	5.68	0.48	25.3	1.08	7.59	6.79
Week 4	13.65	0.26	5.03	0.704	22.9	1.08	7.43	6.95
Week 5	5.4	0.10		0.26	18.7	1.07	7.73	6.83

Table S3. Overview of genes, primer sequences and protocols used in qPCR analysis.

Target gene		Primer sequence	Efficiency (%)	Annealing temperature	Primer Conc (nM)	Reference
act-b	Forward	TCCTCGGTATGGAGTCTTGC	110	60°C	700	[6]
	Reverse	AGCACTGTGTTGGCGTACAG			700	
zo-1	Forward	AGGCTGTGCTGTTCCTCCTA	109.5	60°C	500	[6]
	Reverse	TCCGACGGTAAACATCCTTC			500	
abcb1	Forward	GGCCCAGAACGTGGCTAAC	111.2	55°C	400	[7]
	Reverse	CAGCCATGACAGGTACCACACA			400	
abcc1	Forward	ATCCAGTCCACCATCAGAAC	93.9	55°C	400	[7]
	Reverse	CCTCGGTCCATCACTATCAC			400	
abcc2	Forward	CGCTTCCTCAAACACAACGAG	96.9	55°C	400	[7]
	Reverse	GAACTCTAGACGGATGGCCAG			400	

FIGURES

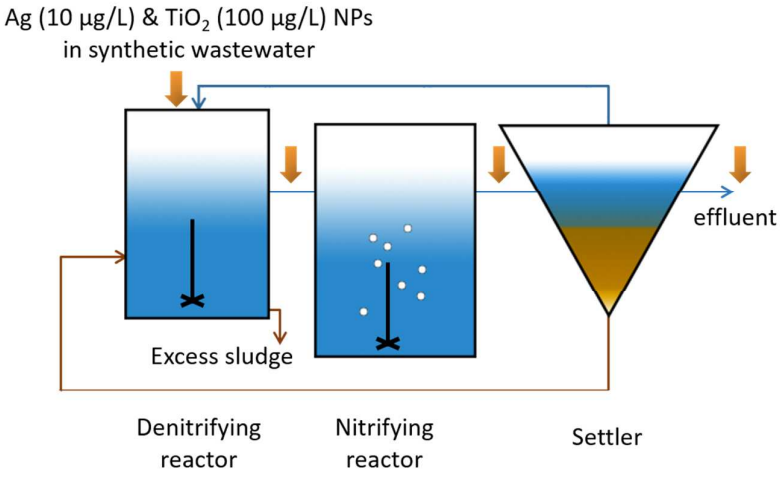

Figure S1. Schematic of the lab-scale WWTP. Orange arrows represent the sampling points for sequential filtration ICP-MS, sp-ICP-MS and STEM.

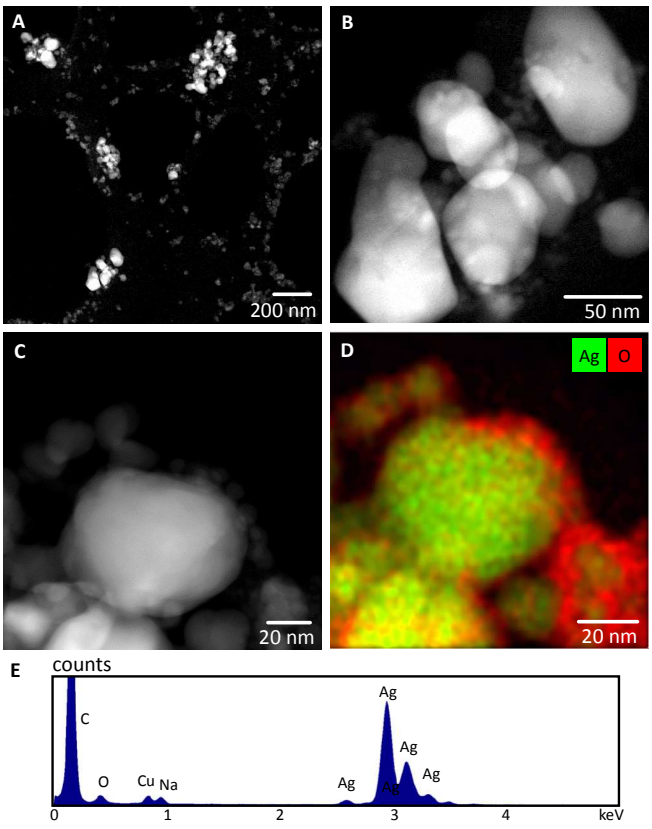

Figure S2. HAADF-STEM images of (A-C) Ag NP (PVP coated) stock dispersions. (C and D) HAADF-STEM images and (D-E) elemental maps of Ag NP (PVP coated) stock dispersions.

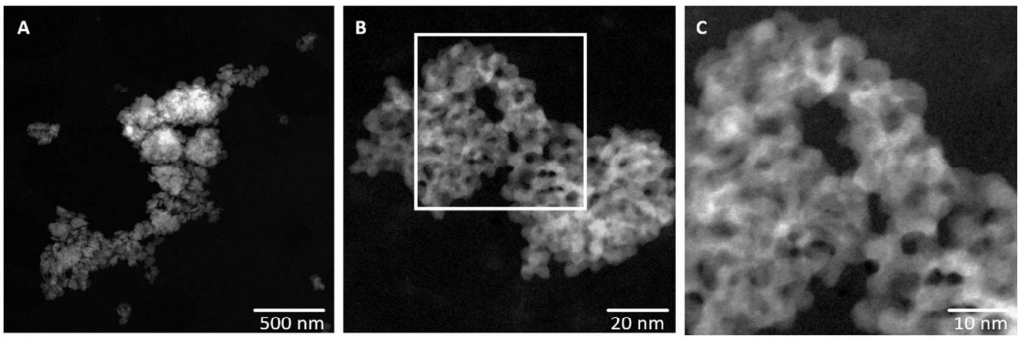

Figure S3. (A-C) HAADF-STEM images of TiO_2 NP stock dispersions.

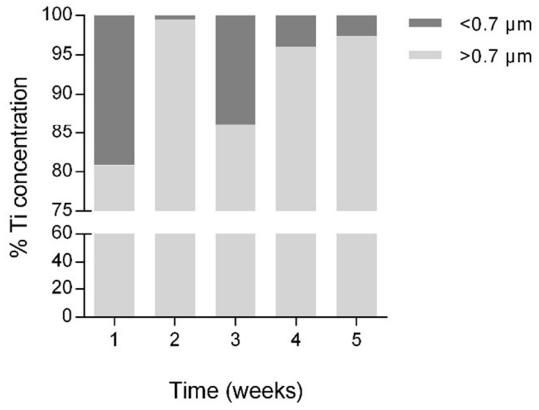

Figure S4. Distribution of the total Ti present in the effluents between different size fractions. Samples for fractionation were collected during the 5 weeks of operation of the system.

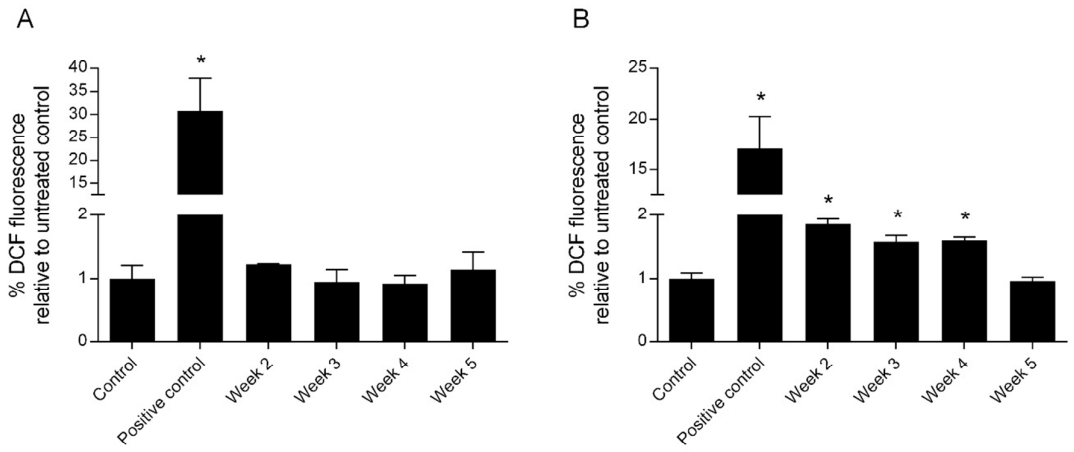

Figure S5. Percentage change in the DCF fluorescence, a proxy for measuring ROS formation, of (A) *S. pseudocostatum* and (B) *R. subcapitata* following 1 h of exposure to the effluents collected in weeks 2-5 and the positive control (10 µM H_2O_2).

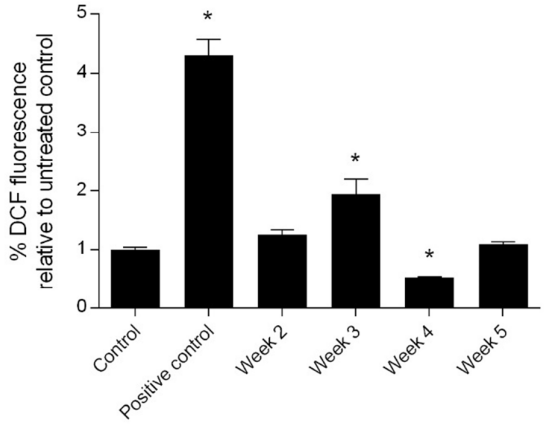

Figure S6. Percentage change in the DCF fluorescence, a proxy for measuring ROS formation, of RTgill-W1 cell line following 1 h of exposure to effluents collected in weeks 2-5 and the positive control (5 µM H_2O_2).

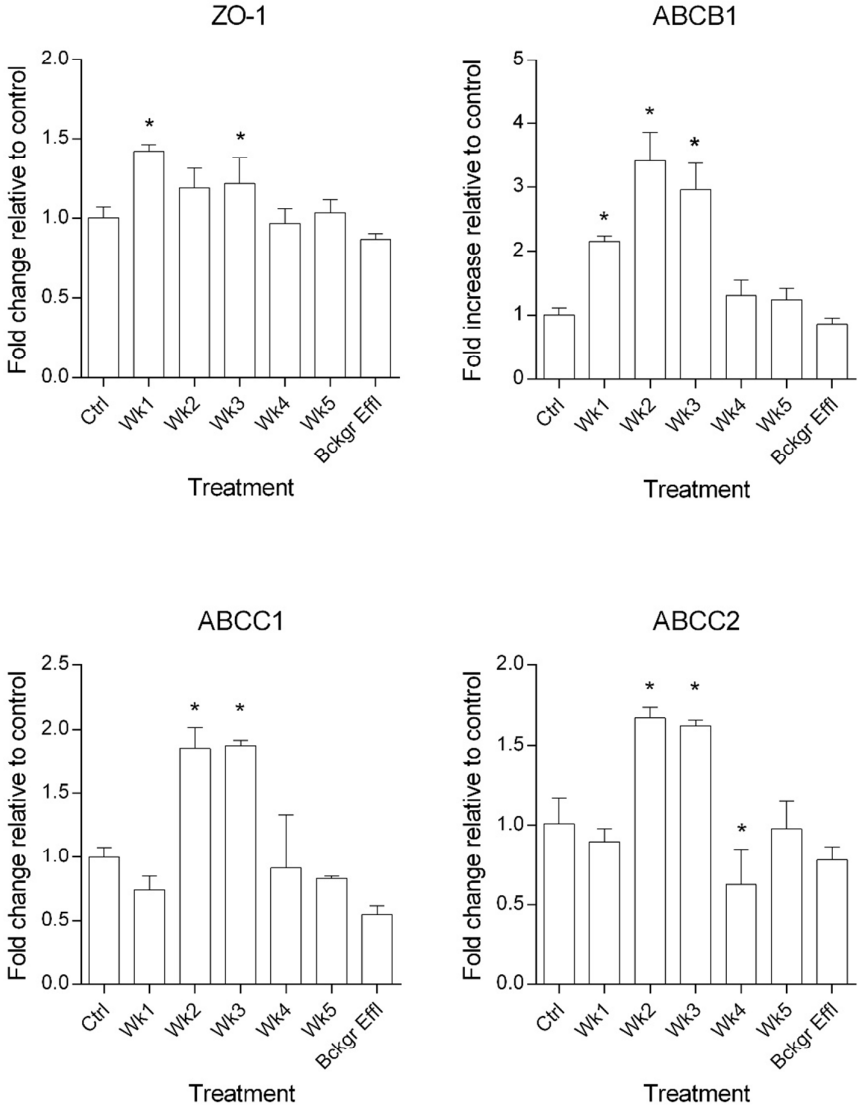

Figure S7. Effects of week 1-5 effluents on the expression of selected genes in the RTgill-W1 cells in transwell-inserts following 24 h exposure. Data are expressed as fold induction relative to the untreated control (L15/ex media) and were normalized to β actin (act-b) gene. Asterisks denote statistical difference compared to control. ZO-1, Zonula occludens; ABCB1, ATP binding cassette subfamily B member 1; ABCC1, ATP binding cassette subfamily C member 1; ABCC2, ATP binding cassette subfamily C member 2.

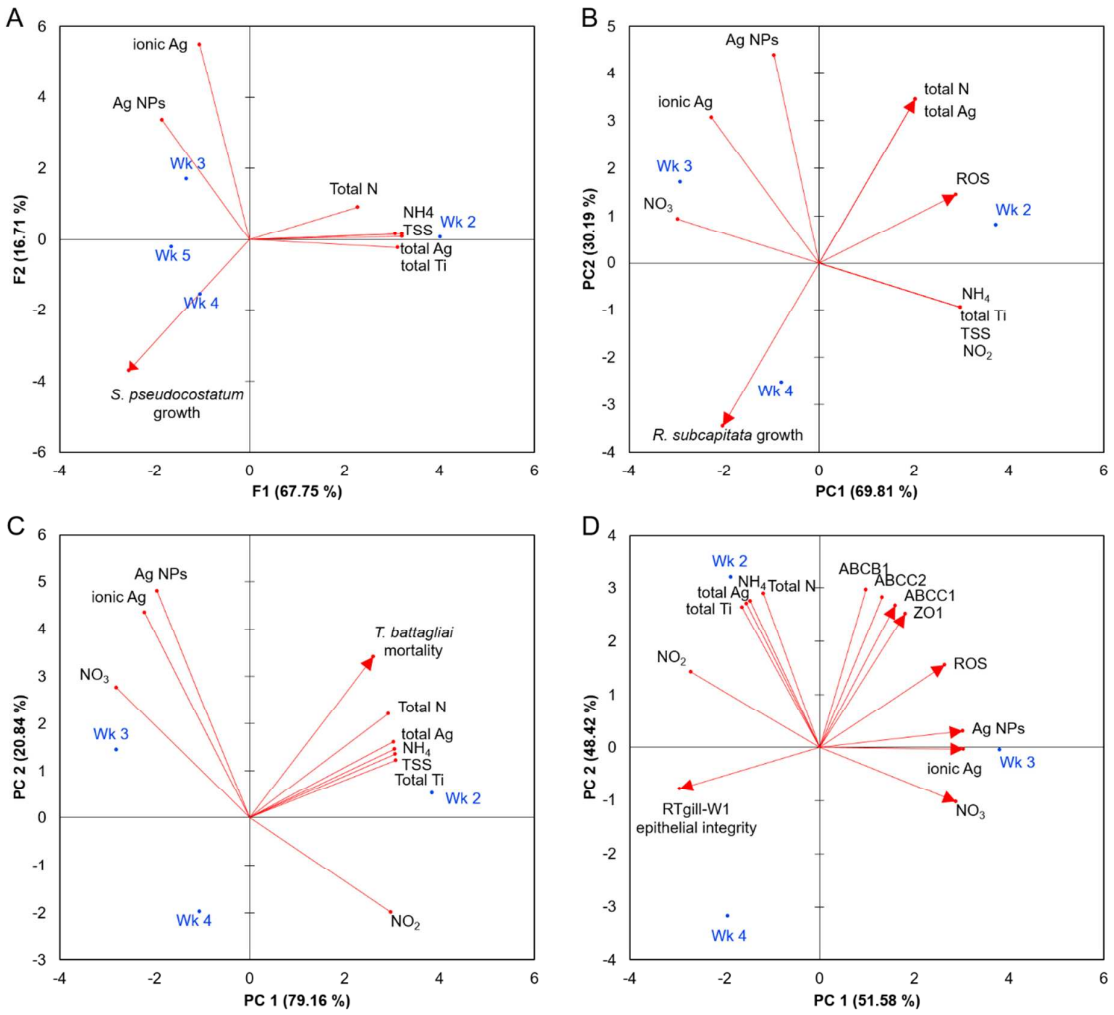

Figure S8. Principal component analysis (PCA) of the physicochemical parameters, Ag and Ti fractionation in the different weeks of effluent collection and the effects obtained with the different bioassays. (A) *S. pseudocostatum* growth, (B) *R. subcapitata* growth, (C) *T. battagliai* mortality and (D) RTgill-W1 epithelial integrity, ROS formation and gene expression.

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