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

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12

## 13 ABSTRACT

14 In this study, a lab-scale wastewater treatment plant (WWTP), simulating biological treatment,  
15 received 10  $\mu\text{g/L}$  Ag and 100  $\mu\text{g/L}$   $\text{TiO}_2$  nanoparticles (NPs) for five weeks. NP partitioning was  
16 evaluated by size fractionation ( $>0.7 \mu\text{m}$ ,  $0.1\text{-}0.7 \mu\text{m}$ ,  $3 \text{ kDa}\text{-}0.1 \mu\text{m}$ ,  $<3 \text{ kDa}$ ) using inductively  
17 coupled plasma mass spectrometry (ICP-MS), single particle ICP-MS and transmission electron  
18 microscopy. The ecotoxicological effects of the transformed NPs in the effluent were assessed  
19 using a battery of marine and freshwater bioassays (algae and crustaceans) and an *in vitro* gill  
20 cell line model (RTgill-W1).  $\text{TiO}_2$  aggregates were detected in the effluent, while Ag NPs ( $0.1$  to  
21  $0.22 \mu\text{g/L}$ ) were associated with S, Cu, Zn. Fractionation showed that  $>80\%$  of Ag and Ti were  
22 associated with the effluent solids. Increased toxicity was observed during weeks 2-3 and the  
23 effects were species-dependent; with marine epibenthic copepods and algae being the most  
24 sensitive. Increased reactive oxygen species formation was observed *in vitro* followed by an  
25 increase in epithelial permeability. The effluent affected the gill epithelium integrity *in vitro* and  
26 impacted defense pathways (upregulation of multixenobiotic resistance genes). To our  
27 knowledge, this is the first study to combine a lab-scale activated sludge WWTP with extensive  
28 characterization techniques and ecotoxicological assays to study the effects of transformed NPs  
29 in the effluent.

30

## 31 INTRODUCTION

32 The production and use of consumer products containing Ag and TiO<sub>2</sub> NPs continues to  
33 increase<sup>1,2</sup> and due to their widespread use and application they can enter sewage streams and  
34 wastewater treatment plants (WWTPs). Their presence in the influent of WWTPs has been  
35 reported in several studies<sup>3-7</sup>. Ag and TiO<sub>2</sub> NPs tend to be associated with particulate matter and  
36 appear to be relatively efficiently removed from the wastewater during primary and secondary  
37 treatment<sup>3,4,6,8,9</sup>, the extent of removal however depends on the design and efficiency of the  
38 operating conditions<sup>6</sup>. Ag-based and TiO<sub>2</sub> NPs have been detected in wastewater effluents<sup>6,9</sup>  
39 making their release in surface waters through effluent discharge possible, which can potentially  
40 be an important exposure route for aquatic organisms in receiving waters.

41 Nanoparticles undergo a combination of physical and chemical transformations in environmental  
42 media (e.g. wastewaters)<sup>10</sup> that may influence their behavior, bioavailability and toxicity<sup>11,12</sup>.  
43 Their behavior may differ from their pristine NP counterparts, thereby making comparisons and  
44 predictions between transformed and pristine NPs difficult. It has been reported that Ag NPs are  
45 sulfidized to various degrees in wastewater streams and during transport to WWTPs<sup>8,13</sup>.  
46 Furthermore, studies using a pilot WWTP fed with municipal wastewater spiked with Ag NPs,  
47 showed a transformation to Ag<sub>2</sub>S while some of the Ag NPs detected in the effluent were still in  
48 the pristine metallic form<sup>14</sup>. Even though most NPs present in the natural environment are likely  
49 to have undergone some form of physicochemical transformation, very few effects studies have  
50 employed transformed NPs<sup>15-17</sup> or NPs in environmentally relevant media such as  
51 wastewaters<sup>12,18,19</sup>. One recent study has shown that Cu NP transformation through a septic tank  
52 led to a lack of toxicity in a zebrafish embryo hatching assay<sup>15</sup>. A decreased toxicity was also  
53 observed for the freshwater amphipod *H. Azteca* exposed to Ag NPs transformed through an

54 activated sludge simulation system<sup>17</sup> while another study showed an increased zebrafish embryo  
55 toxicity in the effluent of a similar system dosed with 4-16 mg/L Ag NPs<sup>20</sup>. Studies using  
56 sulfidized Ag NPs through wastewater treatment processes demonstrated that although Ag<sub>2</sub>S NPs  
57 are less soluble, they can still be bioavailable to different organisms<sup>21,22</sup> and induce toxicity,  
58 though at lower levels compared to pristine Ag NPs<sup>23</sup>. This highlights the need of a better  
59 understanding of the behavior of NPs, their transformation and their toxicity in complex media.

60 It remains a challenging task to detect and quantify NPs at low, but environmentally relevant  
61 concentrations (< μg/L) in complex matrices such as wastewater, effluent, sewage sludge, and  
62 surface waters<sup>24</sup>. As a result, most environmental fate studies and toxicological assessments are  
63 conducted at much higher concentrations than those expected to be found in the environment<sup>20</sup>,  
64 and studies taking into account relevant exposures at more realistic conditions are scarce<sup>15,16</sup>.  
65 There is a need to develop a better understanding of the environmental impact of transformed  
66 NPs at environmentally relevant concentrations<sup>25</sup>.

67 The current study investigates the potential hazard of transformed Ag and TiO<sub>2</sub> NPs through  
68 advanced biological treatment processes present in complex environmental media such as  
69 WWTP effluents at environmentally relevant NP concentrations. A lab-scale pre-denitrifying  
70 WWTP system with pre-conditioned activated sludge was established and continuously fed with  
71 artificial wastewater dosed with 10 μg/L Ag and 100 μg/L TiO<sub>2</sub> NPs for a period of 5 weeks.  
72 The system was combined with a battery of marine and freshwater bioassays and NP  
73 characterization techniques to evaluate the hazard potential of transformed Ag and TiO<sub>2</sub> NPs.  
74 Sequential filtration combined with ICP-MS was applied to characterize the different size  
75 fractions (associated with settling solids, colloidal matter, nanoscale and dissolved). Both marine  
76 (*Skeletonema pseudocostatum*, *Tisbe battagliai*) and freshwater (*Raphidocelis subcapitata*,

77 *Daphnia magna*) organisms (algae and crustaceans) were used as model species to monitor the  
78 toxicity of the transformed NPs present in the effluent during the 5-week dosing period. The  
79 choice of organisms reflects that the behavior of NPs differs in marine and freshwater  
80 environments, the effects may vary depending on the species used<sup>26</sup> as well as the fact that in  
81 many countries the effluent is discharged in both freshwater and marine environments.  
82 Furthermore, an *in vitro* model using the rainbow trout (*Oncorhynchus mykiss*) gill cell line  
83 RTgill-W1 was employed, representing a major interface between the organism and its  
84 environment that is one of the first sites impacted by waterborne chemicals. The model was used  
85 in addition to the standard bioassays for assessment of the effluent with minimal sample  
86 modification during the period of dosing of the WWTP system and cellular responses were  
87 assessed (metabolic activity, epithelial integrity, reactive oxygen species (ROS) formation and  
88 the gene expression of zonula occludens-1 and multixenobiotic resistance genes ABCB1,  
89 ABCC1 and ABCC2).

90

## 91 MATERIALS AND METHODS

92 **Nanoparticles and chemicals.** Polyvinylpyrrolidone (PVP)-coated Ag NPs (Econix 25 nm,  
93 aqueous suspension) were obtained from Nanocomposix (Czech Republic). TiO<sub>2</sub> NPs (NM-101,  
94 primary particles of 5 nm) were obtained from the Joint Research Centre (JRC Repository, Ispra,  
95 Italy) and have been extensively characterized previously<sup>27</sup>. A stock dispersion of TiO<sub>2</sub> NPs in  
96 0.22 µm filtered MilliQ (2.56 mg/ml) was prepared in a Scint-Burk glass vial and sonicated in  
97 ice water for 13 min with a calibrated probe sonicator according to the FP7 EU NANoREG  
98 sonication protocol<sup>28</sup>. The NP stock dispersions were then analyzed with scanning transmission

99 electron microscopy (STEM), single particle (sp-ICP-MS, see sections below) and dynamic light  
100 scattering (DLS) (Supporting Information; SI). AgNO<sub>3</sub> (Sigma-Aldrich) was used as an ionic  
101 control.

102 **Lab-scale wastewater treatment plant.** The lab-scale WWTP was a pre-denitrifying activated  
103 sludge treatment system comprised of a 6.5 L non-aerated denitrifying reactor, an 8 L aerated  
104 nitrifying reactor with automatic temperature (20°C) and pH (7.2) control and a 5.1 L settler (SI;  
105 Figure S1). The activated sludge used in the system was collected from Bekkelaget WWTP,  
106 Oslo, Norway. To adapt the activated sludge to the synthetic medium and to wash out any NPs  
107 transferred to the system together with the initial sludge, the system was continuously fed  
108 (hydraulic retention time ~15 h) synthetic wastewater without NPs for a period of 10 weeks. The  
109 composition and characteristics of the synthetic wastewater and a detailed description of the  
110 system operation and the parameters measured are presented in the SI. Sludge was continuously  
111 removed from the denitrifying reactor to maintain a solids retention time (SRT) of ~15 days.  
112 During the adaptation period effluent samples from the reference system without NPs were  
113 collected weekly and served as “background controls”. After the adaptation period the synthetic  
114 medium was dosed with a continuous supply of 10 µg/L Ag NPs and 100 µg/L TiO<sub>2</sub> NPs to the  
115 denitrifying reactor for a period of 5 weeks. The synthetic wastewater containing Ag and TiO<sub>2</sub>  
116 NPs was freshly prepared every 2-3 days. Effluent samples were collected weekly and used to  
117 evaluate the influence of NP transformation on the battery of bioassays (performed within 48 h  
118 of effluent collection). The COD and total inorganic N removal was 81±8 % and 71±16 %,  
119 respectively (SI).

120 **Ag and TiO<sub>2</sub> NP characterization (STEM/EDS, sp-ICP-MS).** Ag, TiO<sub>2</sub> NP stock dispersions  
121 or effluent samples were imaged using STEM, while elemental point analysis and mapping were

122 performed with energy-dispersive X-ray spectroscopy (EDS). A detailed description of the  
123 STEM-EDS method is presented in the SI.

124 The effluent samples as prepared for STEM were transferred to Eppendorf tubes, vortexed for 30  
125 s, sonicated for 30 min, and then diluted with MilliQ water prior to single particle ICP-MS (sp-  
126 ICP-MS) analysis for particle concentration and size. The sp-ICP-MS analytical protocol and  
127 data analysis (using the single particle RIKILT calculation tool<sup>29</sup>, Wageningen, The Netherlands)  
128 are similar to those described elsewhere<sup>9,29</sup> (detailed description of the sp-ICP-MS method in SI).

129 **Ag and TiO<sub>2</sub> fractionation (filtration, ultrafiltration and ICP-MS).** Samples from the  
130 influent, nitrifying and denitrifying reactors, as well as the effluent (collected from the overflow  
131 of the settler), were collected weekly and fractionated using a series of membranes with  
132 decreasing pore size immediately upon sample collection. The samples were filtered sequentially  
133 through a 0.7 μm filter membrane (glass microfiber GF/F, Whatman, GE Healthcare Life  
134 Sciences), a 0.1 μm membrane (Durapore membrane filter, Millipore) and finally centrifuged  
135 through a 3 kDa cut-off membrane (Amicon Ultra-15, Millipore, 5000g for 1 h) to obtain the  
136 soluble fraction present in the filtrate sample. The 0.7 μm filters were dried at 45°C for 2 h and  
137 kept in microwave tubes until further analysis (solid-associated fraction or particles >0.7 μm).  
138 The solids-associated (>0.7 μm), particulate (0.1-0.7 μm), NP (3 kDa cut-off - 0.1 μm) and the  
139 soluble fraction (3 kDa filtrate) were analyzed by ICP-MS (see SI for details).

140 ***Skeletonema pseudocostatum* growth inhibition assay.** The marine algae were cultured in ISO  
141 media<sup>30</sup> prepared from filtered natural seawater (35 ppt salinity), and maintained at 20°C under  
142 continuous light and shaking according to the ISO 10253 standard. Dilution water used for the  
143 exposure assays was a modified version of the ISO media with a reduced concentration (1/5) of



144 trace elements and EDTA to minimize free metal ion complexation<sup>31</sup> and possible impacts on the  
145 toxicity profile of the effluent. The effluent was spiked with concentrated ISO media stock  
146 solutions to reach the elemental concentrations present in the dilution water. Artificial sea salts  
147 (Coral Pro Salt) were added to reach 35 ppt salinity. Increasing concentrations (5 concentrations:  
148 6.2-100%) of effluent or pristine NPs and AgNO<sub>3</sub> were placed in a 12-well plate (1.35 ml/well,  
149 triplicates). Exponentially growing algae were counted with a hemocytometer and 150  $\mu$ l of  
150  $1 \cdot 10^5$  cells/ ml were added to each well (final algal concentration  $1 \cdot 10^4$  cells/ml). An artificial  
151 seawater control was prepared by spiking artificial sea salts (to achieve 35ppt) into clean dilution  
152 water. Filtered natural seawater with reduced trace elements and EDTA concentrations served as  
153 an untreated control while “background” effluent control was also included. The algal cell  
154 density and growth was assessed daily for 72 h by measuring fluorescence (excitation 530 nm:  
155 emission 685 nm, Victor<sup>3</sup> Multilabel plate reader, PerkinElmer). The specific growth rate  
156 (logarithmic increase in biomass) and the percent growth inhibition over the exposure period was  
157 calculated according to the ISO standard.

158 ***Raphidocelis subcapitata* growth inhibition assay.** The freshwater algae were cultured in EPA  
159 media<sup>32</sup> and maintained at 20°C under continuous light and shaking according to the OECD 201  
160 guideline. The effluent was spiked with concentrated nutrient stock solutions to achieve the same  
161 concentration as the standard media. Trace elements and EDTA were used at a reduced  
162 concentration (1/5). 1.35 ml of increasing concentrations of effluent (5 concentrations: 6.2-  
163 100%), pristine NPs or AgNO<sub>3</sub> were placed in a 12-well plate. Finally, 150  $\mu$ l of algae ( $5 \cdot 10^5$   
164 cells/ml) in exponential growing phase were added per well (final algae concentration  $5 \cdot 10^4$   
165 cells/ml). Dilution water (MilliQ water supplemented with the concentrated stock solutions and  
166 1/5 trace elements-EDTA) served as an untreated control and effluent collected during the

167 stabilization period served as a “background” effluent control. The algal cell number and growth  
168 was measured daily for 72 h (fluorescence measurement, excitation 485 nm: emission 685 nm,  
169 Victor<sup>3</sup> Multilabel plate reader, PerkinElmer).

170 **Effects of effluent on ROS formation (marine and freshwater algae).** Exponentially growing  
171 algae were centrifuged and re-suspended in dilution water to achieve a concentration of  $4 \cdot 10^6$   
172 cells/ml. 25  $\mu$ l of cell suspension was placed in each well of a 96-well plate (final algal  
173 concentration  $1 \cdot 10^6$  cells/ml) and incubated in the dark with 25  $\mu$ l DCFH-DA 20  $\mu$ M (final  
174 concentration 10  $\mu$ M) for 1.5 h under shaking conditions. At the end of the incubation period,  
175 150  $\mu$ l of effluent (serially diluted in dilution water) was added to each well and incubated for 1  
176 h. At the end of the exposure period, DCF fluorescence was measured at wavelengths of 485 nm  
177 excitation and 535 nm emission. H<sub>2</sub>O<sub>2</sub> was used as a positive control.

178 ***Daphnia magna* acute toxicity assay.** Daphnids were maintained in M7 media<sup>33</sup> and fed with *R.*  
179 *subcapitata* every other day. Daphnids <24 h old were used for the assay, which was performed  
180 in 6-well plates as previously described<sup>34</sup> and according to OECD 202 guideline. Five daphnids  
181 per well were used in quadruplicate and were exposed to increasing concentrations of effluent (5  
182 concentrations: 6.25-100%). Moderately hard EPA water was used for dilutions of the effluent<sup>35</sup>.  
183 Daphnids in EPA water served as an untreated control while exposure to effluent collected  
184 during the stabilization period served as a “background” effluent control. The effects of pristine  
185 Ag NPs as well as spiked in background effluent (0.005-0.32 mg/L) were also evaluated.  
186 Daphnid mobility was assessed after 24 and 48 h.

187 ***Tisbe battagliai* acute toxicity assay.** *T. battagliai* were maintained in filtered (0.22  $\mu$ m)  
188 seawater obtained from the outer Oslofjord and fed a mixed diet of *Rhodomonas baltica* and

189 *Isochrysis galbana*. Copepods of  $6 \pm 2$  days old were used for the assay as previously  
190 described<sup>36</sup>. Tests were performed in 12-well plates with 5 animals (4 replicates per treatment) in  
191 each well containing ~4 ml of test solution. Artificial salts (Coral Pro Salt) were added to the  
192 effluent to reach a salinity of 35 ppt, with further dilutions made in the natural seawater used for  
193 culture maintenance. The effects of increasing concentrations of the effluent (5 concentrations:  
194 6.25-100%), Ag NPs (0.08-1.3 mg/L), TiO<sub>2</sub> NPs (0.01-10 mg/L) or AgNO<sub>3</sub> (0.01-0.16 mg/L) in  
195 seawater or spiked in background effluent were assessed after 24 and 48 h of exposure. MilliQ  
196 water spiked with artificial sea salts acted as an artificial seawater control. Natural seawater  
197 served as an untreated control.

198 **RTgill-W1 *in vitro* model in transwell inserts.** The rainbow trout gill epithelial cell line RTgill-  
199 W1<sup>37</sup> was provided by Prof. Kristin Schirmer (EAWAG, Switzerland). Cells were cultured in  
200 Leibovitz's L-15 medium (L-15, Gibco, ThermoFischer Scientific) supplemented with 5% fetal  
201 bovine serum (FBS, Gibco, ThermoFischer Scientific) and 1% gentamicin solution (10 mg/ml,  
202 Sigma-Aldrich), and maintained at 19 °C in an incubator in the absence of CO<sub>2</sub>. The cells were  
203 seeded in 12-well transwell inserts (Millicell Hanging Cell Culture Insert, 1.0 μm, Merck  
204 Millipore) at a concentration of  $1.8 \cdot 10^5$  cells/ml (0.5 ml cell suspension/insert). The basolateral  
205 compartment was filled with 1.5 ml of complete L-15 cell culture medium in a 12-well receiver  
206 plate (Merck Millipore). Cells were allowed to grow for 10 days and form a confluent  
207 monolayer. The media was renewed every other day.

208 **Metabolic activity and epithelial integrity.** On day 10, the cells were exposed for 24 h to  
209 increasing concentrations of the freshly collected effluent from the system (filtered through a 0.2  
210 μm filter; serial dilutions with a dilution factor of 2), the pristine NPs or AgNO<sub>3</sub>. Dilutions were  
211 performed in L15/ex media as previously described<sup>37,38</sup>. Cells in L15/ex media served as an

212 untreated control. At the end of the exposure period the media was removed and replaced with  
213 L15/ex media containing 100  $\mu$ M alamar blue solution. Cells were incubated for 1 h and  
214 fluorescence was measured at wavelengths of 530 nm excitation and 590 nm emission (Victor<sup>3</sup>  
215 Multilabel plate reader, PerkinElmer). The alamar blue solution was then removed and replaced  
216 with 0.1 mg/ml lucifer yellow (LY, Sigma-Aldrich) solution as a marker for paracellular  
217 permeability. The cells were incubated for 2 h before the inserts were removed from the receiver  
218 plates and fluorescence was measured at wavelengths of 485 nm excitation and 535 nm emission  
219 (Victor<sup>3</sup> Multilabel plate reader, PerkinElmer).

220 **Quantitative real time PCR (qPCR).** After exposure of the RTgill-W1 cells in transwell  
221 inserts, the exposure medium was removed, the cells were washed in PBS and were collected  
222 with 300  $\mu$ l RLT plus buffer (Qiagen) supplemented with 1% mercaptoethanol. Total RNA was  
223 extracted using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions and  
224 as previously described<sup>39</sup>. The RNA purity and concentration were determined using a Nanodrop  
225 ND1000 spectrophotometer while RNA integrity was determined with an Agilent Bioanalyzer  
226 RNA 6000 nano series kit (Agilent technologies, USA). The qPCR was performed as previously  
227 described<sup>39</sup> (protocol details can be found in SI).

228 **Effects of effluent on ROS formation (*in vitro*).** RTgill-W1 cells were seeded in 96-well plates  
229 at a concentration of  $5 \cdot 10^5$  cells/ml (100  $\mu$ l cell suspension/well). After 24 h, the media was  
230 removed and fresh media containing 25  $\mu$ M DCFH-DA in L15/ex media was placed in each well  
231 (100  $\mu$ l solution/well). After a 1 h incubation, the DCFH-DA solution was removed and replaced  
232 with increasing concentrations of effluent (5 concentrations: 6-100%), Ag NPs, TiO<sub>2</sub> NPs or  
233 AgNO<sub>3</sub> diluted in L15/ex. Fluorescence was measured after 1 and 2 h of exposure at wavelengths  
234 of 485 nm excitation and 535 nm emission. H<sub>2</sub>O<sub>2</sub> was used as a positive control.

235 **Statistical analysis.** Statistical analysis was performed with GraphPad Prism 6 (GraphPad  
236 Software, La Jolla, CA 92037, USA). Values are expressed as means  $\pm$  standard deviation.  
237 Significant differences between the different treatments and control were analyzed with one-way  
238 analysis of variance (ANOVA) followed by Dunnet's multiple comparison test or nonparametric  
239 Kruskal-Wallis test followed by Dunn's multiple comparison test. Statistical significance was  
240 defined at  $p < 0.05$ . Dose-response curves,  $EC_{10}$  and  $EC_{50}$  values were obtained with GraphPad  
241 Prism 6 (GraphPad Software, La Jolla, CA 92037, USA) using a logistic four-parameter model.  
242 Principal component analysis (PCA) of the parameters and effects observed with the different  
243 bioassays was performed with XLSTAT 2018 (SI).

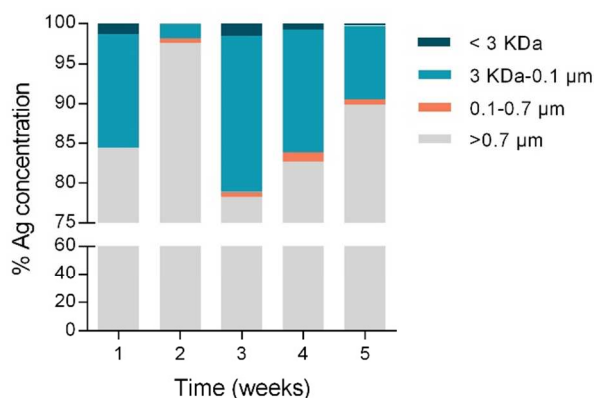
## 244 **RESULTS AND DISCUSSION**

245 **Ag and TiO<sub>2</sub> Nanoparticle characterization.** The physicochemical properties determined for  
246 the Ag and TiO<sub>2</sub> NP stock dispersions in MilliQ water are summarized in the SI (Figures S2-3,  
247 Table S1). The Ag NPs were spherical with a mean diameter of  $26.5 \pm 0.7$  nm and  $58.8 \pm 0.19$   
248 nm according to sp-ICP-MS and DLS measurements, respectively. DLS and sp-ICP-MS analyses  
249 showed an average TiO<sub>2</sub> aggregate size of  $640.7 \pm 9.2$  and  $278 \pm 15$  nm, respectively. STEM  
250 imaging indicated that TiO<sub>2</sub> NPs were porous and formed large aggregates consisting of  
251 individual particles below 10 nm, confirming previous reports on this material<sup>27</sup>. In synthetic  
252 wastewater and seawater TiO<sub>2</sub> aggregates of  $969 \pm 19$  nm and  $1375 \pm 76.7$  nm, respectively were  
253 measured with DLS (SI; Table S1). Ag NPs in synthetic wastewater, seawater and the exposure  
254 media used in the bioassays ranged from  $57.3 \pm 0.17$  to  $59.5 \pm 0.18$  nm as measured with DLS,  
255 suggesting a stability of the PVP-coated Ag NPs in the different media. The higher ( $\sim 2x$ ) particle  
256 size obtained for both pristine Ag NPs and TiO<sub>2</sub> with DLS is probably related to the inherent  
257 properties of the instrument, light scattering techniques such as DLS require higher

258 concentrations that can result in aggregation that could influence the analytical signal<sup>40</sup>. With sp-  
259 ICP-MS low concentration levels can be detected in more complex or natural environmental  
260 samples. Therefore, multiple analytical techniques are necessary especially for low NP  
261 concentrations in environmental samples.

262 **Ag and TiO<sub>2</sub> NP transformation in the lab-scale WWTP.** Sequential filtration and ICP-MS  
263 analysis of the individual effluent fractions showed that >80% of the Ag and Ti measured was  
264 associated with suspended solids (>0.7 μm fraction) present in the effluent samples (Figure 1,  
265 Figure S4). The highest concentrations of both total Ag and Ti were observed in effluents from  
266 weeks 2 and 5. The Ti levels in the fraction >0.7 μm ranged from 0.9-24.2 μg/L, with the highest  
267 concentration measured at week 2. The dissolved Ag concentration was in the range of 0.005-  
268 0.021 μg/L (Table 1). The highest dissolved Ag concentrations were observed in effluents  
269 collected after 1 and 3 weeks of NP dosing, and corresponded to 7-8% of the total Ag measured  
270 during those weeks. The Ag concentration present in the NP fraction ranged from 0.1-0.22 μg/L,  
271 with the highest concentrations measured in the effluent samples collected in weeks 1, 3 and 5  
272 (0.22, 0.14 and 0.17 μg/L, respectively). The Ti present in the 0.1 μm and 3 KDa fractions could  
273 not be distinguished and quantified separately, therefore the values are reported as Ti >0.7 μm  
274 and <0.7 μm. A previous study with sequencing batch reactors showed that a significant fraction  
275 of Ag was associated with colloidal material (below 0.45 μm)<sup>41</sup> and biosolids in the sludge and  
276 effluent of a pilot WWTP<sup>14</sup>.

277



278

279 **Figure 1.** Effluent characterization and distribution of the total Ag present in the effluent of the  
 280 lab-scale WWTP system during the 5 weeks of continuous dosing of the system.

281

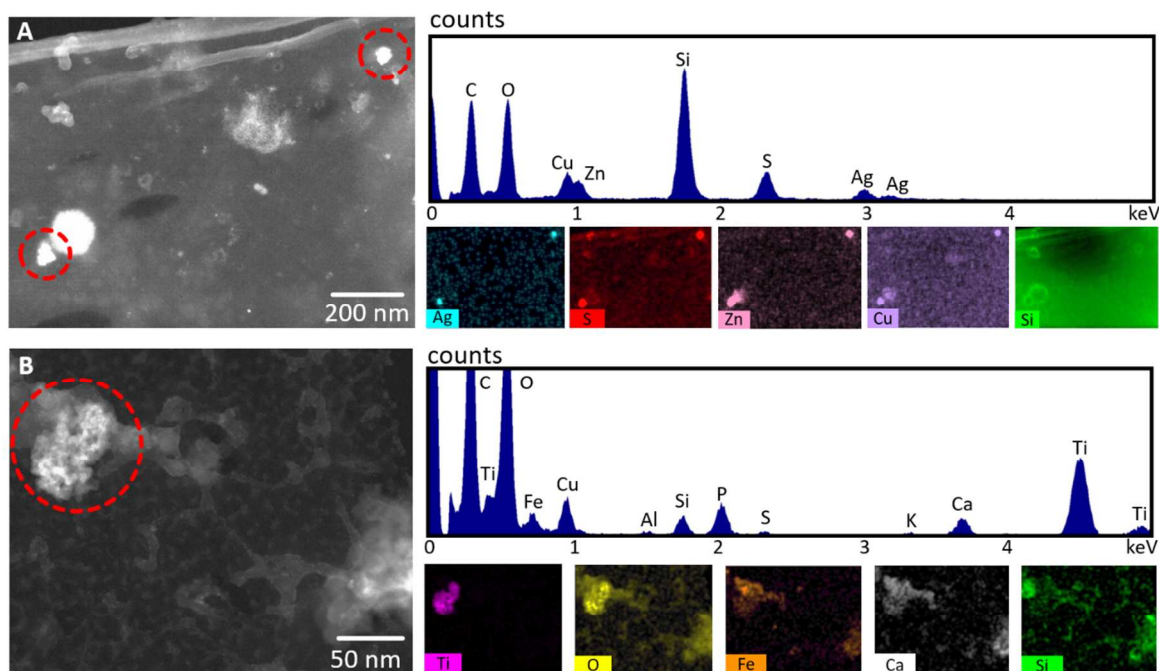
282 **Table 1.** Ag and Ti concentrations ( $\mu\text{g/L}$  or  $\mu\text{g/g}$  effluent suspended solids) in each effluent  
 283 fraction during the 5 weeks of operation and continuous dosing of the lab-scale WWTP system.

Effluent Sample	Ag concentration								Ti concentration			
	Total		>0.7 $\mu\text{m}$		nano-Ag		3 KDa filtrate		>0.7 $\mu\text{m}$		<0.7 $\mu\text{m}$	
	$\mu\text{g/L}$	$\mu\text{gAg/gSS}$	$\mu\text{g/L}$	$\mu\text{gAg/gSS}$	$\mu\text{g/L}$	$\mu\text{gAg/gSS}$	$\mu\text{g/L}$	$\mu\text{gAg/gSS}$	$\mu\text{g/L}$	$\mu\text{gTi/gSS}$	$\mu\text{g/L}$	$\mu\text{gTi/gSS}$
wk 1	0.74	47.34	0.51	32.21	0.22	13.82	0.02	1.31	0.90	57.50	0.14	8.67
wk 2	5.99	72.15	5.84	70.41	0.11	1.28	<0.005	0.06	24.20	291.52	0.13	1.55
wk 3	0.72	66.28	0.56	51.88	0.14	12.98	0.01	1.01	1.00	92.17	0.16	14.81
wk 4	0.65	47.90	0.54	39.60	0.10	7.37	<0.005	0.37	2.50	183.15	0.10	7.50
wk 5	1.80	333.22	1.62	299.75	0.17	30.59	<0.005	0.93	5.40	999.30	0.15	27.04

284

285 The effluent collected during the 4<sup>th</sup> week of system operation was analyzed by STEM in  
 286 combination with EDS to determine both the presence and transformation of Ag and  $\text{TiO}_2$  NPs.  
 287 Electron microscopy images showed the presence of particles with high mass (bright contrast),  
 288 while EDS analysis indicated that Ag-rich particles were associated with S, Cu and Zn (Figure  
 289 2A). STEM also showed the presence of  $\text{TiO}_2$  polycrystalline aggregates ( $\sim 50$  nm) (Figure 2B)

290 comprised of primary particles below 10 nm which were similar to the initially dosed particles.  
291 The association of Ag present in WWTP with elements such as Cu, Zn and S is in accordance  
292 with previous studies reporting the presence of Ag particles associated with S in sludge<sup>14,42</sup> and  
293 effluent samples<sup>14</sup>. It has recently been shown that secondary nano-sized Ag particles of  
294 approximately 20 nm diameter associated with S from organic or inorganic source are formed  
295 from dissolved silver from Ag NPs (80 nm, PVP coated) in batch systems with wastewater  
296 effluent and mixed liquor<sup>10</sup>.



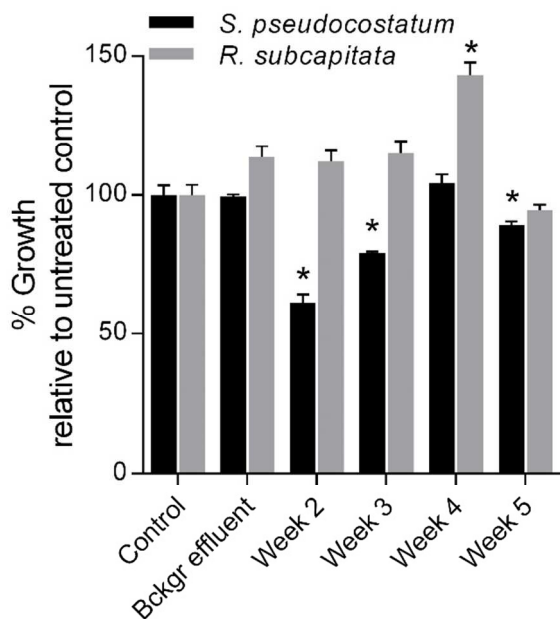
297  
298 **Figure 2.** STEM images of (A) Ag-rich and (B) TiO<sub>2</sub> particles from the lab-scale WWTP,  
299 together with sum spectra of the encircled regions and elemental maps. Particles were detected in  
300 the effluent collected during the 4<sup>th</sup> week of dosing and operation of the system.

301



302 Single particle ICP-MS analysis of effluent samples collected during the 5 weeks of operation of  
303 the system confirmed the presence of Ag and TiO<sub>2</sub> NPs, indicating they occurred within the size  
304 ranges 20.5-31.6 nm and 110.9-124.8 nm, respectively (SI; Table S1). Sp-ICP-MS is a very  
305 promising technique for the identification and quantification of metallic NPs in complex  
306 matrices<sup>43</sup>, including wastewater and effluents<sup>44-46</sup>. The technique has low detection limits<sup>47</sup> and  
307 requires highly diluted samples that are very relevant for environmental samples, as well as when  
308 realistic exposures are to be studied. However, distinction between Ag complexes and species or  
309 Ag bound colloids cannot be made<sup>45</sup>.

310 **Effects of effluents on algal growth and ROS formation.** A 20-40% growth inhibition of the  
311 marine algae, *S. pseudocostatum*, was observed upon exposure to effluents at the highest effluent  
312 concentration (100%; Ag and Ti exposure concentrations of 6 and 24 µg/L, respectively), with  
313 effluent from week 2 showing the strongest effect (40% growth inhibition relative to untreated  
314 control) (Figure 3). However, results from the DCFH-DA assay indicated that no formation of  
315 ROS occurred for any of the tested effluents (SI; Figure S5). Exposure to the background effluent  
316 alone did not result in any significant effect on algal growth. These concentrations are below the  
317 respective no effect concentration (NOEC) values obtained for *S. pseudocostatum* in this study (1  
318 mg/L and 10 mg/L for Ag and TiO<sub>2</sub> NPs). This suggests that the presence of solids and elevated  
319 NH<sub>4</sub> concentrations (3.3 mg/L) contribute to the observed effects and not just the total Ag and Ti  
320 present in the effluents (Table S2, Figure S8). Differences in toxicity of Ag NPs aged in crude  
321 and final wastewater have been reported and decreased toxicity was related to the sample  
322 physicochemical parameters and increased complexity<sup>48</sup>.



323

324 **Figure 3.** Percentage growth of *S. pseudocostatum* (black bars) and *R. subcapitata* (grey bars)

325 exposed to effluents collected in weeks 2-5 (100% and 50% effluent concentration for *S.*

326 *pseudocostatum* and *R. subcapitata*, respectively) and the background effluent. Algal growth

327 inhibition was determined after 72 h of exposure. Asterisks denote statistical significance at

328  $p < 0.05$ .

329

330 In contrast to the inhibitory effects of the effluent on *S. pseudocostatum* growth, there was

331 evidence of hormetic effects in the freshwater algae, *R. subcapitata* exposed to effluent

332 concentrations  $< 50\%$ . These effects were most apparent after exposure to effluent collected from

333 week 4 and showed significant stimulatory effects on growth compared to the control (40%

334 increase in growth compared to control) (Figure 3). The stimulatory effects in *R. subcapitata*

335 growth were accompanied by a significant increase in the ROS formation (1.6-1.9-fold compared

336 to untreated control) (SI; Figure S5) and increased cell aggregation (observed by microscopy,

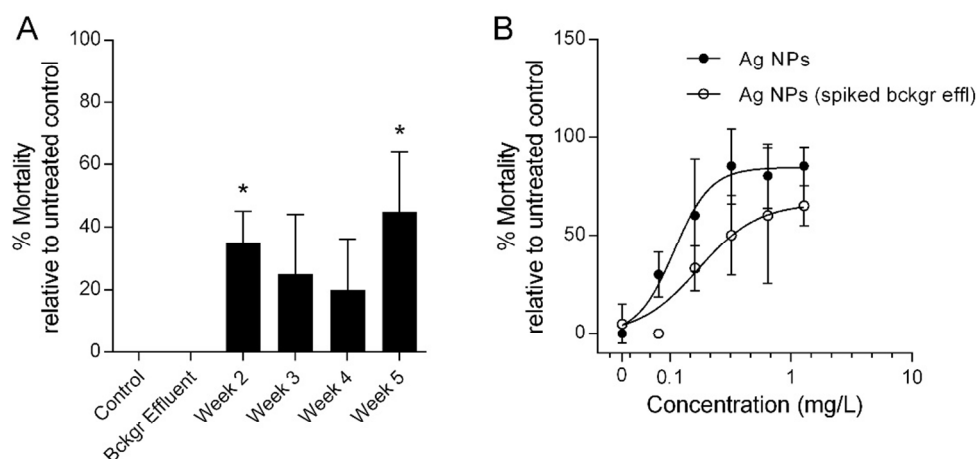
337 data not shown). The ROS formation was positively correlated with the total Ag and Ti  
338 concentration, total N and suspended solids present in the effluents (Figure S8). A similar  
339 response of cell aggregation has been previously reported upon exposure of the green algae  
340 *Chlamydomonas reinhardtii* to CuO-polystyrene core-shell NPs<sup>49</sup> and *Chlorella vulgaris* and  
341 *Dunaliella tertiolecta* to Ag NPs<sup>50</sup>. It has been suggested that cell aggregation is a defense  
342 mechanism that decreases the amount of exposed surface to xenobiotics. Moderate stress and low  
343 ROS levels can lead to hormetic effects that can in turn induce the defense system<sup>51</sup>. The results  
344 from the current study indicate that responses to the effluent exposure are species-dependent,  
345 possibly due to differences in cell size, surface area and cell wall composition. Studies with  
346 green algae and cyanobacteria exposed to Ag NPs have also shown differences in cell viability  
347 and ROS response between species attributed to different biological properties and the  
348 production of extracellular polymeric substances<sup>52</sup>. Moreover, the NP behavior depends on the  
349 media composition that can result in different responses, TiO<sub>2</sub> aggregates of 1369 nm were  
350 observed in the presence of Cl in the higher ionic strength media of *S. pseudocostatum* compared  
351 to 650 nm aggregates in *R. subcapitata* media while the Ag NPs seemed to be stable in both  
352 media. The formation of insoluble AgCl(s) and dissolved silver chloride species depends on the  
353 Cl/Ag ratio<sup>53</sup> which could further explain differences in effects observed between the freshwater  
354 and marine algae.

355 **Effects of effluents on *T. battagliai* and *D. magna*.** Exposure to effluents collected weekly  
356 during the operation of the system led to a 20-45% increase in mortality of *T. battagliai* (at 100%  
357 effluent concentration), while no effect was observed from the background effluent (Figure 4A).  
358 The highest significant mortality was observed upon exposure to effluents collected in weeks 2  
359 and 5 (35 and 45% mortality compared to untreated control, respectively). Spiking the

360 background effluent with increasing concentrations of Ag NPs elicited a reduction in toxicity at  
361 the lowest Ag NP concentration (0.08 mg/L) compared to pristine Ag NPs, but still caused a  
362 significant increase in mortality at most concentrations (Figure 4B). Spiking the background  
363 effluent also resulted in a 1.9x increase in the EC<sub>50</sub> value compared to the pristine Ag NPs (0.09  
364 and 0.17 mg/L, respectively) although the EC<sub>50</sub> values were not statistically significant (Figure  
365 4B). TiO<sub>2</sub> NPs did not have any effect on mortality at any of the concentrations tested (0.01-10  
366 mg/L).

367 Although the total Ag concentration in the effluents (5.99 µg/L or 72.15 µg/gSS) exceeded the  
368 NOEC for Ag NPs (0.005 mg/L), and was at a similar level to the EC<sub>10</sub> obtained in this study  
369 (0.0076 mg/L), no adverse effects on daphnid mobility were observed following 48 h exposure to  
370 either the effluents or the background effluent. Spiking of the background effluent with  
371 increasing concentrations of Ag NPs led to a significant decrease in mobility, but resulted in an  
372 16x increase in the EC<sub>50</sub> value compared to the pristine Ag NPs (0.16 and 0.0098 mg/L,  
373 respectively) (Figure 5). TiO<sub>2</sub> NPs did not affect daphnid mobility.

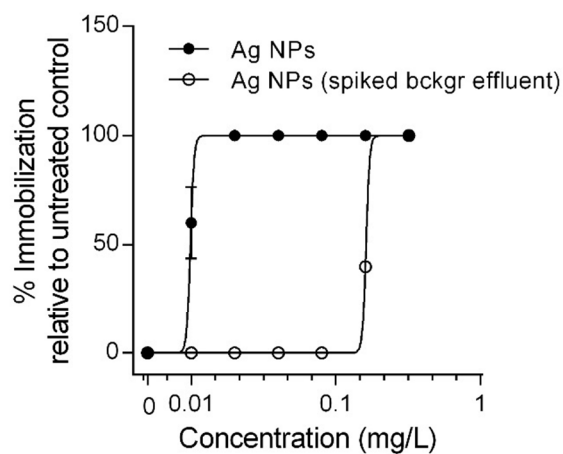
374



375

376 **Figure 4.** Percentage mortality of *T. battagliai* following 48 h exposure to (A) effluents collected  
 377 in weeks 2-5 and (B) increasing Ag NP concentrations as received or spiked in the background  
 378 effluent. Asterisks denote statistical significance at  $p < 0.05$ .

379



380

381 **Figure 5.** Percentage immobilization of *D. magna* juveniles following 48 h exposure to  
 382 increasing Ag NP concentrations and Ag NP-spiked background effluent. Background effluent  
 383 was collected during the system stabilization period (prior to spiking). No effects of effluents  
 384 collected in weeks 2-5 and background effluent were observed.

385

386 A clear reduction in the toxicity of Ag NPs to *D. magna* was observed when exposed to the  
387 effluent collected from the lab-scale WWTP system (containing transformed Ag NPs) compared  
388 to pristine Ag NPs. Unlike *D. magna*, the marine copepod *T. battagliai* exhibited a clear  
389 response following exposure to the week 2-5 effluents (statistically significant in weeks 2 and 5).  
390 The difference in response between the two species may result from a combination of NP  
391 behavior in more complex WWTP effluents and differences in the feeding behavior of the two  
392 organisms. *D. magna* is a planktonic filter feeding organism<sup>54</sup> while *T. battagliai* is an  
393 opportunistic feeding epibenthic organism<sup>55</sup>. Therefore, *T. battagliai* is likely to be directly in  
394 contact with particles associated with effluent solids that may settle out during the exposure  
395 period. *T. battagliai* are non-selective grazers as well as filter feeders and feed on suspended  
396 particles along with detritus that settles out of the water column<sup>56</sup>. These differences in feeding  
397 habit could explain the increased sensitivity of the copepods compared to daphnids when  
398 exposed to the WWTP effluent. In contrast to this *D. magna* was 10x more sensitive to pristine  
399 Ag NPs compared to *T. battagliai* (Figure 4 and 5). Therefore, the complete absence of effects in  
400 *D. magna* exposed to any of the collected effluents reinforces the idea that NPs present in the  
401 effluent are associated with the solids settling on the bottom of the vessels, reducing direct  
402 exposure and ingestion by the daphnids.

403 To further confirm this, *T. battagliai* and *D. magna* were exposed to the background effluent  
404 spiked with increasing concentrations of Ag NPs which led to decreased toxicity relative to the  
405 pristine Ag NPs. However, for *T. battagliai* the EC<sub>50</sub> value only increased 2 times, whereas for  
406 *D. magna* the EC<sub>50</sub> value increased 16 times. This indicates the presence of solids in the effluent,  
407 as well as the potential formation of precipitates, reduces the bioavailability of the Ag NPs to the

408 daphnids compared to *T. battagliai*. This is in accordance with previous studies where reduced  
409 toxicity of AgNO<sub>3</sub> spiked into untreated effluent was observed for the freshwater green algae *C.*  
410 *reinhardtii*<sup>19</sup> and the protective effects of background effluent were observed towards Cu  
411 interference with zebrafish hatching<sup>15</sup>. Furthermore, a decrease in the bioavailability of Ag from  
412 AgNO<sub>3</sub>-exposed algae (*C. reinhardtii*) was observed in wastewaters, and suggested to be due to  
413 the presence of ligands<sup>12</sup>. It has been previously demonstrated that sulfidation<sup>53</sup>, the presence of  
414 natural organic matter<sup>57</sup> and thiol- or selenide-containing compounds such as cysteine<sup>58</sup> can  
415 reduce the Ag NP dissolution rate and lead to protective effects due to Ag<sup>+</sup> complexation and  
416 decreased bioavailability<sup>59,60</sup>, partially explaining the reduced toxicity of Ag NPs spiked in  
417 background effluent. The differences in EC50 increase trends of Ag NP-spiked background  
418 effluent compared to pristine Ag NPs between the 2 organisms can also be attributed to  
419 differences in media composition and ionic strength. The formation of AgCl precipitates in  
420 media with high Cl content such as in seawater can further impact the Ag<sup>+</sup> availability and  
421 subsequent toxicity<sup>53,61</sup>. Species-specific differences were related to the degree of Ag NP  
422 sulfidation, the exposure route and species sensitivity<sup>53</sup>.

423 Therefore, the effects of Ag NPs observed in the current study are considered organism-  
424 dependent, with (epi)benthic organisms having the highest exposure risk due to directly ingesting  
425 sedimented and aggregated NPs or NPs bound to effluent solids. In addition, the media  
426 composition can impact the NP speciation and behavior leading to increased TiO<sub>2</sub> NP  
427 aggregation and formation of silver chloride species in media of increasing ionic strength.

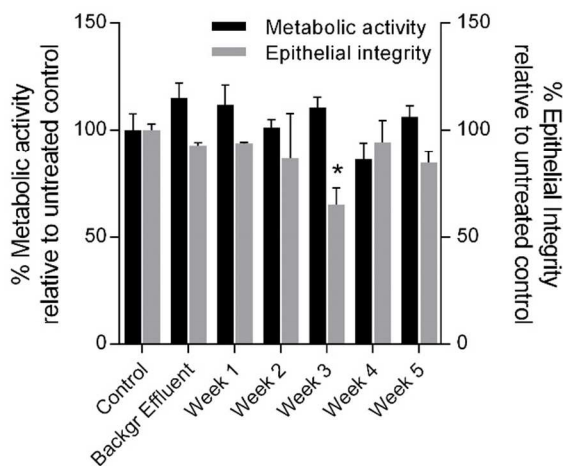
428 **Effects of effluents on RTgill-W1 cells.** The *in vitro* fish gill cell line model was employed in  
429 the current study as the gill is a key site for xenobiotic uptake and it is continuously exposed to  
430 water-borne contaminants<sup>62</sup>. Furthermore, the gills express enzymes involved in xenobiotic

431 metabolism and transport. Exposure to the 1-5 week effluents did not cause a statistically  
432 significant decrease in the metabolic activity of RTgill-W1 cells in transwell inserts (Figure 6). A  
433 40% decrease in the epithelial integrity (Figure 6), which coincided with a 2-fold increase in  
434 ROS formation (Figure S6), was observed upon exposure to effluent from week 3. However, no  
435 statistically significant effect was observed for any of the other effluents and no effect was  
436 observed for the “background” effluent for either endpoint. Previous studies have shown that  
437 primary fish gill cell cultures in permeable filter supports can tolerate apical water and varying  
438 osmotic conditions<sup>63</sup>, river water<sup>64</sup>, detect bioreactive metals<sup>64,65</sup>, and have been used to study the  
439 uptake and transport of Ag NPs<sup>66</sup>. In the current study, it has proven to be a good model system  
440 for whole effluent toxicity testing without the need for sample modification or alteration of the  
441 water chemistry prior to exposure. However, the concentrations of Ag and TiO<sub>2</sub> NPs measured in  
442 the effluent are considered too low to fully account for the effects observed in the metabolic  
443 activity and epithelial integrity assays. Given the complexity of the wastewater effluent, it  
444 appears that the combination of the presence of Ag NPs, ionic Ag and additional stressors such  
445 as NO<sub>3</sub> contribute to the overall response observed (Figure S8).

446



447



448

449 **Figure 6.** Percentage change in metabolic activity (left Y axis, black bars) and epithelial integrity  
450 (right Y axis, grey bars) of RTgill-W1 cells following exposure to effluents collected in weeks 1-  
451 5. Asterisks denote statistical significance at  $p < 0.05$ .

452

453 As effects were observed on the epithelial integrity, and because the gill is a site of xenobiotic  
454 uptake and detoxification, the effects of the effluents on the gene expression of zonula  
455 occludens-1 (ZO-1) tight junction protein and multixenobiotic resistance genes in RTgill-W1  
456 cells were studied. The ZO-1 gene was selected due to the decreased epithelial integrity observed  
457 in the paracellular permeability assay. Results showed ZO-1 mRNA levels were elevated after  
458 exposure to effluents collected on week 1 and 3 (SI; Figure S7). Previous studies have shown  
459 that the RTgill-W1 cells express functional tight junctions that can respond to certain  
460 modulators<sup>67</sup>. In the current study, the RTgill-W1 cell model in transwell inserts showed an  
461 increased paracellular permeability followed by an increase in ZO-1 expression upon exposure to  
462 week 3 effluent, suggesting an impact on the epithelial integrity and a compromised barrier  
463 function. Moreover, the DCFH-DA assay indicated exposure to the week 3 effluent led to a 2-

464 fold increase in ROS formation, suggesting a ROS-induced compromised epithelial integrity. It  
465 has previously been shown that oxidative stress can lead to a disruption of tight junctions in  
466 MDCK canine kidney cells<sup>68</sup>.

467 The multixenobiotic resistance (MXR) mechanism mediated by ATP binding cassette  
468 transporters is an important mechanism of defense against xenobiotics, which functions by  
469 extruding them or their metabolites out of the cell. The transporters are localized in tissues with a  
470 barrier function or involved in secretion and absorption, they transport a wide variety of  
471 compounds across cell membranes and it has recently been shown that NPs, including Ag NPs,  
472 can interfere with the MXR system<sup>69,70</sup>. Due to their importance in cellular defense against  
473 xenobiotics, the multixenobiotic resistance genes ABCB1, ABCC1, ABCC2 were also  
474 investigated in the current study. Exposure to the effluents led to increased mRNA levels of  
475 ABCB1, ABCC1 and ABCC2 transporters, with ABCB1 (the most responsive) exhibiting  
476 increased expression levels in response to effluents from weeks 1-3 (3.4-fold increase upon  
477 exposure to effluent week 2) (SI; Figure S7). These results indicate an interference with the  
478 defense mechanism and potentially compromised protection against xenobiotics. The  
479 contribution of other trace elements and other unidentified stressors present in the effluent to the  
480 observed effects cannot be excluded. It also remains to be determined whether this observed  
481 change in gene expression also leads to transporter functional changes.

482 **Environmental implications.** The combination of a lab-scale WWTP with detailed fractionation  
483 approaches, characterization techniques (TEM, sp-ICP-MS, sequential filtration/ICP-MS), a  
484 battery of marine and freshwater bioassays and an *in vitro* gill cell line model allowed the effects  
485 of transformed NPs to be investigated. This study shows that Ag NPs are transformed through  
486 simulated biological WWTP processes to particles associated with S, Cu and Zn. The resulting

487 hazard cannot be predicted based on exposures made in simplified media or determined by  
488 measuring the NP concentration and the dissolved fraction since the effluent is complex with  
489 additional stressors (e.g suspended solids,  $\text{NH}_4$ ) either exacerbating or mitigating the effects  
490 depending on the organism, endpoint and media used. The transformed particles appeared to  
491 have a greater impact on epibentic copepods suggesting that they were still bioavailable despite  
492 their transformation. Differences in responses in marine vs freshwater algae and crustaceans  
493 highlight the importance of the media composition in the NP speciation that can lead to species-  
494 specific responses. The study reinforces the need to use multiple test species representing  
495 different environments and exposure routes, bioassays and endpoints to gain clearer  
496 understanding of the potential hazards of low level realistic concentrations of transformed  
497 nanomaterials and multiple stressors in environmental media of increased complexity. The  
498 results highlighting the difference in toxicity of pristine and transformed particles, emphasize the  
499 need for future studies using a broader range of weathered or transformed NPs in relevant  
500 exposure scenarios to provide a more accurate understanding of their potential impacts. The  
501 combination of complementary analytical techniques (TEM, sp-ICP-MS, sequential  
502 filtration/ICP-MS) was useful for the detection and characterization of low NPs concentrations in  
503 complex environmental matrices. Our results demonstrated that Ag and  $\text{TiO}_2$  NPs show a strong  
504 association with solids, suggesting the potential for terrestrial organisms' exposure through  
505 biosolid<sup>21,42,71</sup> application. Based on these conclusions future studies should focus on the effects  
506 of transformed NPs associated with the biosolids on terrestrial organisms and the factors  
507 contributing to species-specific responses.

508

509 ASSOCIATED CONTENT

510 **Supporting Information.** Additional information is provided for the synthetic wastewater  
511 composition, the lab-scale WWTP description and operation (and schematic; Figure S1), sample  
512 preparation description for STEM/EDS and sp-ICP-MS, mass balance calculations for Ag and  
513 TiO<sub>2</sub> NPs, DLS measurements of TiO<sub>2</sub> and Ag NPs stock dispersions in MilliQ water, synthetic  
514 wastewater, seawater and exposure media, sp-ICP-MS measurements of NP stock dispersions  
515 and effluents (Table S1), characteristics of the effluents collected in weeks 1-5 (Table S2) and an  
516 overview of genes, primer sequences and protocol used for qPCR (Table S3). In addition, TEM  
517 images of Ag and TiO<sub>2</sub> NPs stock dispersions are provided (Figure S2, S3), fractionation of Ti  
518 (Figure S4), effects of effluents on *S. pseudocostatum* and *R. subcapitata* ROS formation (Figure  
519 S5), effects of effluents on RTgill-W1 ROS formation (Figure S6), gene expression (Figure S7)  
520 and principal component analysis (PCA) of the physicochemical parameters and effects observed  
521 in the different bioassays (Figure S8).

522

## 523 AUTHOR INFORMATION

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528 to the final version of the manuscript.

529

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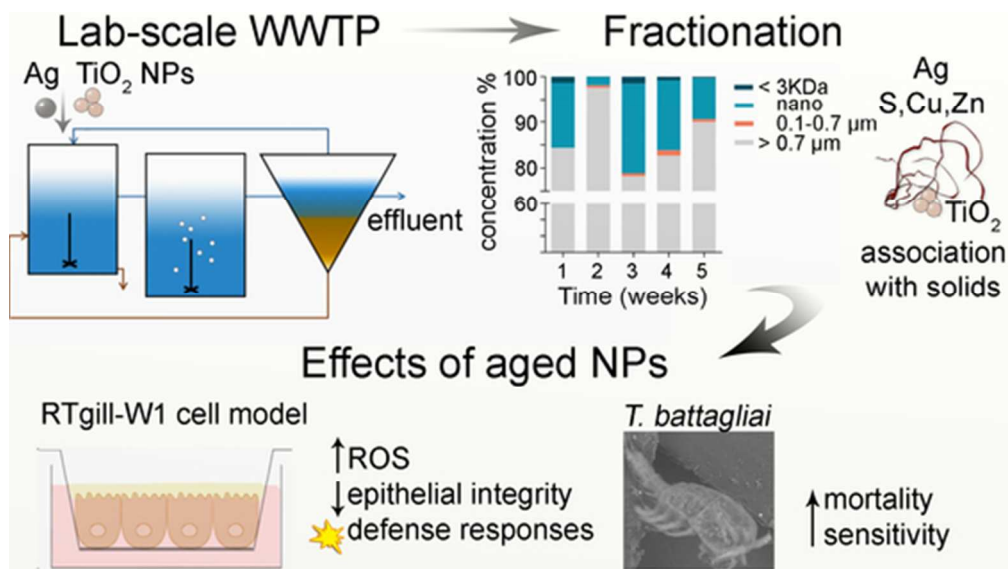
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Graphical abstract

47x26mm (300 x 300 DPI)