

## Wastewater-aged silver nanoparticles in single and combined exposures with titanium dioxide affect the early development of the marine copepod *Tisbe battagliai*

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13

## 14 ABSTRACT

15 In this study, the effects of aged Ag and TiO<sub>2</sub> NPs, individually and as a mixture, in wastewater  
16 relative to their pristine counterparts on the development of the copepod nauplii (*Tisbe*  
17 *battagliai*) were investigated. NP behavior in synthetic wastewater and seawater was  
18 characterized during aging and exposure. A delayed development and subsequent mortality  
19 was observed after 6 days of exposure to aged Ag NPs, with a 2-fold decrease in EC<sub>50</sub> (316  
20 μg/L) compared to pristine NPs (EC<sub>50</sub> 640 μg/L) despite the similar dissolved Ag  
21 concentrations measured for aged and pristine Ag NPs (441 μg/L and 378 μg/L, respectively).  
22 In co-exposures with TiO<sub>2</sub> NPs, higher dissolved Ag levels were measured for aged NPs (238.3  
23 μg/L) relative to pristine NPs (98.57 μg/L). Co-exposure resulted in a slight decrease (15%) in  
24 the Ag NP EC<sub>50</sub> (270 μg/L) with a 1.9-fold increase in Ag NP retained within the organisms  
25 after depuration (2.82% retention) compared to Ag NP single exposures as measured with sp-  
26 ICP-MS suggesting that the particles are still bioavailable despite the heteroaggregation  
27 observed between Ag, Ti NPs and wastewater components. This study shows that the presence  
28 of TiO<sub>2</sub> NPs can affect the stability and toxicity of Ag NPs in complex media that cannot be  
29 predicted solely based on ionic, total or nanoparticulate concentrations and the need for  
30 studying NP interactions in more complex matrices is highlighted.

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## 33 INTRODUCTION

34 Silver (Ag) and titanium dioxide (TiO<sub>2</sub>) nanoparticles (NPs) are used in numerous industrial  
35 applications and the number of consumer products incorporating Ag and TiO<sub>2</sub> NPs has  
36 increased over recent years<sup>1,2</sup>. Both Ag and TiO<sub>2</sub> NPs are released into wastewater streams  
37 during the life cycle of such products, reaching wastewater treatment plants (WWTPs). As a  
38 result, NPs in consumer products are likely to be released into the aquatic environment through  
39 effluent discharges or through direct release into surface waters during product use (e.g.  
40 sunscreens). Several studies have shown the presence of nanosized Ag and TiO<sub>2</sub> particles in  
41 WWTPs<sup>3,4</sup>, effluents and surface waters<sup>5</sup>.

42 In different environmental matrices, NPs can undergo a number of environmental  
43 transformations (aging), including changes in agglomeration/aggregation, oxidation,  
44 dissolution, precipitation of other forms, surface modification and interaction with (in)organic  
45 species/ligands<sup>6,7</sup>. Aging and transformation of NPs in environmental media typically leads to  
46 NPs with altered physicochemical properties that differ from their pristine forms. This can  
47 result in subsequent changes in particle behavior, leading to differences in fate, uptake and  
48 subsequent effects that are difficult to predict from information on the pristine particles alone.  
49 Laboratory exposures with well-dispersed pristine NPs are therefore not expected to represent  
50 realistic environmental conditions for effects assessment. Several studies have stressed the  
51 need to assess the effects of NPs in more relevant and complex matrices such as wastewaters  
52 and effluents. It has also been suggested that aging of NPs prior to ecotoxicological assessment  
53 can minimize further transformation during exposures and allow particle behavior to be studied  
54 under more environmentally realistic conditions<sup>8</sup>. Despite this, the majority of ecotoxicological  
55 studies are still conducted in simplified/standardized media, with only a limited number of  
56 exposure studies incorporating transformed materials being reported<sup>7,9-13</sup>.

57

58 Several studies have described the specific changes or transformation that may occur to specific  
59 NP materials in various environmental matrices. The chemical transformation of Ag NPs to  
60 nanoscale Ag<sub>2</sub>S species has been reported in WWTPs<sup>14</sup>. In Cl<sup>-</sup>-rich media, oxidative dissolution  
61 is followed by the formation of different AgCl species, which have been shown to remain  
62 bioavailable to aquatic organisms<sup>15</sup>. A study using CuO NPs showed that transformation within  
63 a laboratory septic tank led to decreased toxicity in the zebrafish embryo hatching assay due to  
64 formation of non-bioavailable Cu species<sup>10</sup> while a decreased bioavailability and toxicity of  
65 wastewater-borne Ag NPs was previously shown for rainbow trout<sup>16</sup>. In contrast, effluents from  
66 Ag NP-spiked lab-scale sewage treatment plant exhibited higher toxicity to zebrafish embryos  
67 than the pristine NPs<sup>11</sup>. Another study using a lab-scale WWTP spiked with Ag and TiO<sub>2</sub> NPs  
68 (co-exposure) for 5 weeks has shown that Ag particles were still present in the effluent and  
69 despite the observed Ag NP transformation, they were found to impact the survival of the  
70 marine copepod *Tisbe battagliai* and led to increased oxidative stress levels, increased  
71 paracellular permeability and altered defense responses to the RTgill-W1 cell line model<sup>9</sup>. It  
72 has recently been shown that Ag NPs were still bioavailable to soil microorganisms and  
73 terrestrial isopods, despite undergoing sulfidation<sup>17,18</sup>.

74 It has been highlighted that the behavior and fate of NPs in environmental media depends on  
75 the size/aggregate size, concentration and media composition<sup>8,19-21</sup>. Therefore, in depth  
76 characterization of the exposure conditions is essential for an improved understanding of  
77 particle interactions with organisms and for assessing the environmental hazard. Moreover,  
78 different NP types will most likely co-exist in WWTPs and receiving environmental  
79 compartments and their interaction can result in further transformation and altered

80 bioavailability and toxicity, yet studies investigating the combined effects of multiple NPs are  
81 very scarce<sup>9,22,23</sup>.

82 The aim of the current study was to elucidate differences in the toxicity of pristine and  
83 wastewater-aged Ag NPs and TiO<sub>2</sub> NPs and their mixture using sensitive life stages of the  
84 epibenthic marine copepod *T. battagliai*. A pre-aging step in synthetic wastewater (4h) was  
85 incorporated to account for rapid transformations previously demonstrated for NPs in  
86 wastewater. Pristine Ag NPs and TiO<sub>2</sub> NPs were used 'as received' for comparison. The NP  
87 behavior and characteristics during the aging process and during the exposure period was  
88 studied using a combination of imaging and analytical techniques, including dynamic light  
89 scattering (DLS) and Laser Doppler electrophoresis (size and agglomeration/aggregation,  
90 surface charge), Ultraviolet–visible spectroscopy (UV-Vis, stability in different media) and  
91 inductively coupled plasma mass spectrometry (ICP-MS, Ag dissolution and concentration  
92 determination). Single particle (sp)-ICP-MS combined with an enzymatic digestion method  
93 was used to determine the NP concentration retained in the organisms at the end of the exposure  
94 period. The effects of aged vs pristine NPs on the early life stage development of *T. battagliai*  
95 was studied over 6 days of exposure. *T. battagliai* was chosen as a model organism due to their  
96 importance and ecological significance in food webs, short life cycle, distinctive developmental  
97 stages, relatively high sensitivity and the relevance of epibenthic organisms to NP exposure<sup>9</sup>.

## 98 MATERIALS AND METHODS

### 99 **Nanoparticle preparation.**

100 Polyvinylpyrrolidone (PVP) coated Ag NPs (Econix 25 nm, Nanocomposix, Czech Republic)  
101 were supplied as aqueous dispersion (no other dispersant was present), while TiO<sub>2</sub> NPs (NM-  
102 101, 5 nm primary particles, Joint Research Centre Repository, Ispra, Italy) were supplied in  
103 powder form and dispersions prepared as previously described<sup>9</sup> and according to the FP7 EU

104 NANoREG sonication protocol<sup>24</sup>. Briefly, TiO<sub>2</sub> NP stocks (2.56 mg/ml in 0.22 μm filtered  
105 MilliQ water) were prepared by sonicating in an ice water bath for 13 min using a calibrated  
106 probe sonicator. The NP stocks were characterized with scanning transmission electron  
107 microscopy (STEM), single particle ICP-MS (sp-ICP-MS) and dynamic light scattering (DLS,  
108 Zetasizer nano ZS Malvern Instruments, UK) as described previously<sup>9</sup>. To prepare the exposure  
109 dispersions, the stock dispersions were either diluted directly in filtered (0.22 μm) natural  
110 seawater (referred to as pristine NPs), or were aged in synthetic wastewater (SWW) before  
111 being diluted in seawater (referred to as aged NPs). The concentrations used for the aging  
112 process were 10 mg/L for Ag NPs, 100 mg/L for TiO<sub>2</sub> NPs and these were combined for the  
113 NP mixture (10 and 100 mg/L Ag and TiO<sub>2</sub> NPs, respectively). The Ag NP and TiO<sub>2</sub> NP  
114 concentrations used in the current study are higher than the levels currently being measured in  
115 WWTP influents and surface waters. The justification for the selected concentrations was the  
116 limitations with DLS characterization in complex matrices. In complex matrices such as  
117 wastewater or SWW there is interference caused by the high particle background concentration.  
118 A ten times higher concentration of TiO<sub>2</sub> NPs relative to Ag NPs was used due to the higher  
119 concentrations of TiO<sub>2</sub> NPs commonly found in environmental samples and WWTPs.

#### 120 **NP aging in synthetic wastewater and characterization.**

121 *Exposure dispersion preparation.* To represent the rapid transformation of NPs that occurs in  
122 wastewater, Ag NPs, TiO<sub>2</sub> NPs and the NP mixture were aged in 600 ml SWW (SWW  
123 composition is described in detail in the Supplementary Information section; SI) in 1 L  
124 borosilicate glass jars (Flocculator 2000, Kemira Oyi, Finland) prior to use in exposure studies.  
125 The samples ( $n=3$ ) were stirred rapidly (250 rpm) for 30 seconds to ensure homogeneous  
126 dispersion of the NP in the SWW, and were subsequently stirred slowly (50 rpm) for 4 h at 20  
127 ± 2°C. Samples were intermittently taken at time points 0 h, 1 h, 2 h and 4 h to characterize the

128 particle behavior in SWW over time. SWW-aged (4h) NP dispersions of Ag NPs, TiO<sub>2</sub> NPs  
129 and the NP mixture used for exposure experiments were prepared by tenfold dilution in filtered  
130 natural seawater. Pristine NP exposure dispersions were similarly prepared by diluting  
131 respective stocks directly in filtered natural seawater. Exposure dispersions (n=3) of pristine  
132 and aged Ag NPs (1 mg/L), TiO<sub>2</sub> NPs (10 mg/L) and the NP mixture (1 and 10 mg/L Ag and  
133 TiO<sub>2</sub> NPs, respectively), were characterized with UV-vis and DLS at 0 h, 24 h, 48 h, and 72 h,  
134 corresponding to the exposure duration (exposure dispersion renewals at t=72 h). At the end of  
135 the 4 h aging process, the exposure dispersions at the highest nominal exposure concentration  
136 were quantified using ICP-MS to help interpret data from the effect studies on the development  
137 of the *T. battagliai*. Ag NP characterization and quantification methodology by STEM (in  
138 synthetic wastewater) and sp-ICP-MS is described in the Supporting Information (SI).

139 *Particle size and zeta potential.* The particle size and size distribution,  
140 agglomeration/aggregation, particle count and surface charge (zeta potential) during aging and  
141 exposures were evaluated using a Zetasizer instrument (NanoZS Malvern Instruments, UK).

142 *Particle concentration via UV-Vis.* To determine NP concentrations with UV-vis (UV-mini  
143 1240, Shimadzu, Japan), absorbance peaks for each particle type were determined by spectral  
144 scans (190-800 nm). Calibration curves for Ag NP and TiO<sub>2</sub> NP dilution series (MilliQ-water)  
145 were prepared using peak absorbance values. The samples were resuspended manually  
146 immediately prior to the analysis.

147 *Total and dissolved Ag and Ti concentration-ICP-MS.* Samples for total Ag and Ti content  
148 determination were obtained as explained in the previous section. The presence of dissolved  
149 species of Ag and Ti was obtained by centrifugal ultrafiltration of the samples through a 3 kDa  
150 cut-off membrane at 5000 g for 1 h (Amicon Ultra-15 Centrifugal Filter Unit, Millipore). The  
151 samples were acidified by ultrapure HNO<sub>3</sub>:HF (5:1 v/v) and analyzed with ICP-MS (Agilent



152 8800, triple Quadrupole). For quality control, blanks and certified reference material (NCS DC  
153 73349) were included in the analysis.

154 *Sp-ICP-MS analysis.* Sub-samples of Ag NPs during aging and exposures were stored frozen  
155 at -20°C until analysis. Samples were vortexed for 30s and diluted with MilliQ water (to reach  
156 an Ag concentration of approximately 5 ppt) prior to analysis. The analytical protocol and data  
157 analysis (single particle RIKILT calculation tool<sup>25</sup>) was performed as previously described<sup>9,25</sup>  
158 (detailed method description in SI).

### 159 **Effects on *T. battagliai* nauplii development.**

160 *T. battagliai* cultures were maintained in natural seawater (0.22 µm filtered, 35 ppt salinity)  
161 obtained from the outer Oslofjord and were fed a mixed algal diet of *Rhodomonas baltica* and  
162 *Isochrysis galbana*. The developmental assay was performed as previously described<sup>26</sup> with  
163 slight modifications. Gravid females were separated from the cultures and nauplii <12 h post-  
164 release were individually placed in the well of a 24-well plate containing 2 ml of test solution  
165 (10 replicates per concentration). A concentration representing 10% of the aged stocks in  
166 seawater was used as the highest exposure concentration (corresponding to nominal  
167 concentrations of 1 mg/L Ag NPs, 10 mg/L TiO<sub>2</sub> NPs and 1 and 10 mg/L Ag and TiO<sub>2</sub> NPs,  
168 respectively in the NP mixture). The effects of increasing concentrations of Ag NPs (0.06,  
169 0.125, 0.25, 0.5, 1 mg/L), TiO<sub>2</sub> NPs (0.6, 1.25, 2.5, 5, 10 mg/L) and the corresponding NP  
170 mixture were assessed over an exposure duration of 6 days. AgNO<sub>3</sub> (0.003, 0.006, 0.0125,  
171 0.025, 0.05 mg/L) was used as an ionic control. The exposure dispersions were prepared from  
172 the highest nominal exposure concentration and a concentrated cell suspension of *R. baltica*  
173 was added to reach 2·10<sup>5</sup> cells/ml (final concentration) prior to distribution to the exposure  
174 wells. Exposure solutions containing *R. baltica* (2·10<sup>5</sup> cells/ml final concentration) were  
175 renewed on day 3. A SWW control (10% in seawater as per the other highest nominal exposure

176 concentrations) and a natural seawater control were included. The pH in controls and  
177 treatments were monitored at the beginning and end of the exposures and ranged from 8.0 to  
178 8.2 while the dissolved oxygen levels ranged from 7.29 to 7.65 mg/L. The developmental stage  
179 of the copepods was evaluated daily and mortality or other abnormalities were recorded. The  
180 naupliar developmental rate (% nauplii reaching the copepodid stage at the end of the study  
181 compared to the controls) was calculated for each treatment.

### 182 **NP uptake and detection in *T. battagliai*.**

183 To study the uptake of particles, organisms exposed to 0.006 mg/L AgNO<sub>3</sub>, 0.125 mg/L Ag  
184 NPs and the mixture of Ag NPs (0.125 mg/L) and TiO<sub>2</sub> NPs (1.25 mg/L) were transferred to  
185 clean natural seawater at the end of the exposure period (day 6) and allowed to depurate for 24  
186 h. The concentrations were chosen based on the highest concentration that did not exert an  
187 effect. At the end of the depuration period, 5 individuals were pooled and transferred to  
188 Eppendorf tubes. They were washed twice with a solution containing N-Acetyl-L-cysteine  
189 (NAC, Sigma Aldrich, final concentration 0.5 mM in MilliQ water) to remove any loosely  
190 bound ionic Ag or NPs on the surface of the organisms and the samples were stored at -20°C  
191 until further processing. The digestion process was performed as previously described<sup>25</sup>, but  
192 slightly modified and optimized for *T. battagliai* used in this study. Briefly, 0.2 ml of digestion  
193 buffer (10 mM Tris buffer, 0.1% Triton X and 1 mM calcium acetate, pH 9) was added to each  
194 tube containing 5 copepods and the samples were sonicated for 1 h in a sonication bath. The  
195 samples were then enzymatically digested by adding 1 µL of Proteinase K (Merck-Millipore,  
196 final concentration 100 µg/mL) to each tube and incubating the samples overnight at 37°C. The  
197 samples were then sonicated for 15 min in a sonication bath and diluted in MilliQ water prior  
198 to sp-ICP-MS analysis (analysis was performed within 1 hour of sample dilution). In order to  
199 assess the impact of the digestion process on the NP size, samples of the digestion buffer spiked

200 with Ag NPs according to the procedure described above were also analyzed with sp-ICP-MS  
201 and resulted in particles of  $27 \pm 1.4$  nm. Although there was no visible debris after the  
202 enzymatic digestion, incomplete digestion of the exoskeleton cannot be excluded. Therefore,  
203 any NPs remaining (after the washing steps with NAC) associated with the exoskeleton were  
204 not taken into account in the sp-ICP-MS measurement.

### 205 **Statistical Analysis.**

206 Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, La Jolla,  
207 CA). Values are expressed as means  $\pm$  standard deviation and the exposure experiments were  
208 repeated 2 to 3 times. Significant differences in concentrations between aged and pristine  
209 particles and between treatments and controls were analyzed with one-way ANOVA followed  
210 by Sidak's or Dunnet's multiple comparison test. Dose response curves and EC50 values were  
211 obtained with GraphPad Prism 6 (GraphPad Software, La Jolla, CA 92037) using a logistic  
212 four-parameter model.

## 213 **RESULTS AND DISCUSSION**

214 **Stock dispersion characterization.** DLS analysis showed that the pristine Ag NPs had an  
215 average hydrodynamic (z-ave) size of  $66 \pm 0.1$  nm, while the TiO<sub>2</sub> NPs had a z-ave size of  
216  $358 \pm 11$  nm (Table 1), which is in accordance with our previous studies showing the presence  
217 of TiO<sub>2</sub> aggregates consisting of NP below 10 nm<sup>9,27</sup>. According to sp-ICP-MS analysis, the  
218 average size of Ag NPs in the stock dispersion was  $24 \pm 3$  nm (Figure S1). Due to the aggregate  
219 size of the TiO<sub>2</sub> particles in the stock dispersion, sp-ICP-MS was only performed for Ag NP  
220 stock dispersions.

221 **Nanoparticle dispersion characterization during aging and exposure.** An increase in the z-  
222 ave was observed for both particles during aging in SWW, resulting in values of  $381 \pm 44$  nm

223 for the Ag NPs and  $511 \pm 7.5$  nm for the TiO<sub>2</sub> NPs after 4 h (Table 1). This may at least partly  
 224 be attributed to the presence of particles present in SWW, which had a z-average of  $536 \pm 34$   
 225 nm (data not shown). A similar trend of increasing size was also previously reported for Ag  
 226 NPs during aging in crude wastewater<sup>28</sup>. A previous study has shown that PVP-coated Ag NPs  
 227 were rapidly transformed to Ag(0) in influent wastewater (after 4 min of spiking), suggesting  
 228 that a modification/loss of the surface coating occurs<sup>29</sup>. Despite the fact that this can vary  
 229 between different PVP-coated NPs, a potential modification of the surface coating in the  
 230 present study cannot be excluded and may have contributed to the aggregation observed in  
 231 SWW immediately after spiking.

232

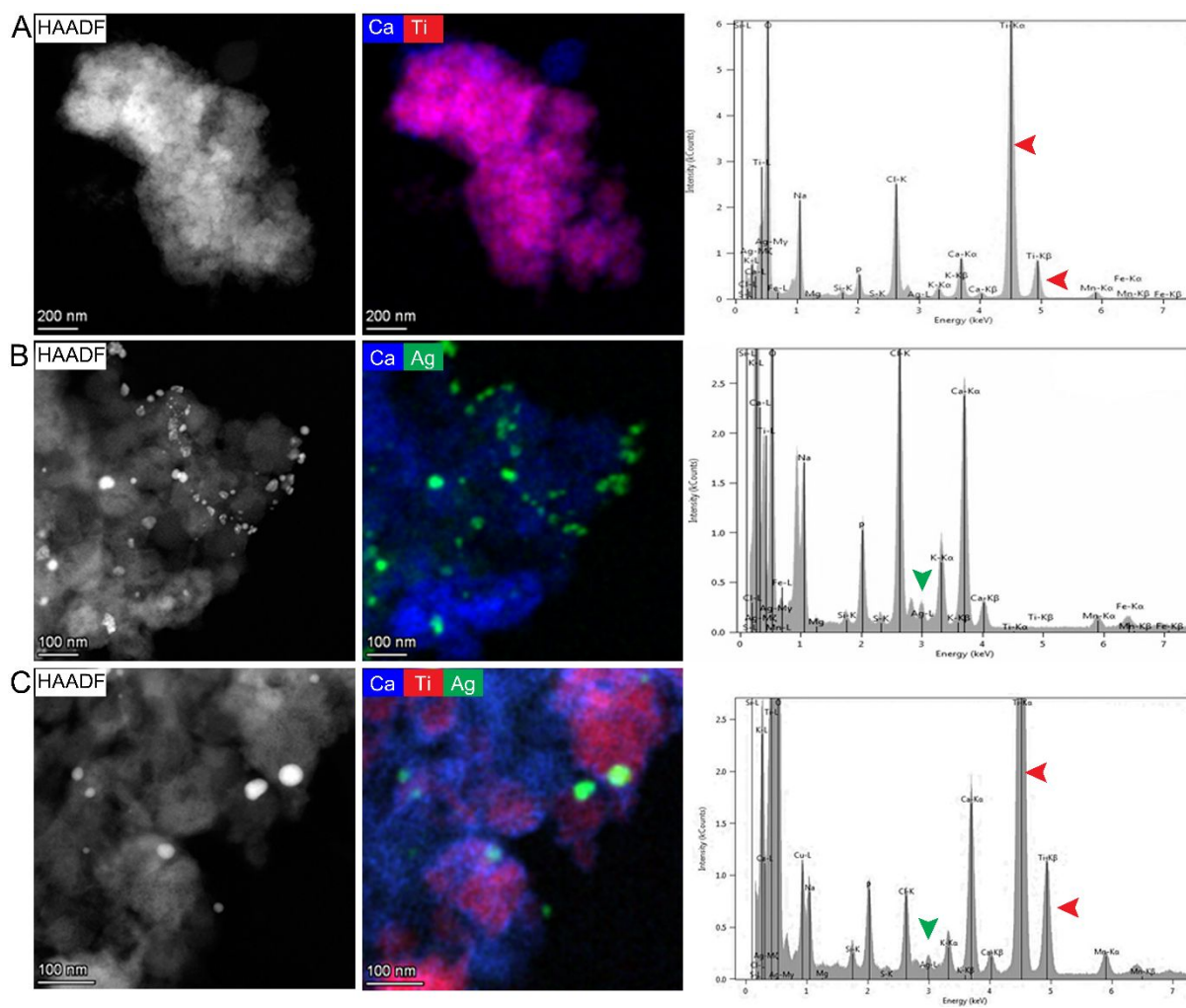
233 **Table 1.** Characterization data for Ag NPs, TiO<sub>2</sub> NPs and the NP mixture at either: 0 h (start)  
 234 and 4 h (end) of aging (in synthetic wastewater; SWW), or during exposure in natural seawater  
 235 as aged or pristine at 0h (start of exposure) and 72h (exposure solution renewal). Values  
 236 presented are the average  $\pm$  standard deviation of triplicates.

	Stock	SWW		Pristine NP in seawater		Aged NP in seawater		
		during aging		during exposure		during exposure		
		0 h	4 h	0 h	72 h	0 h	72 h	
Ag NPs	z average (nm)	66 $\pm$ 0.1	391 $\pm$ 30	381 $\pm$ 44	75 $\pm$ 2.5	90 $\pm$ 11	268 $\pm$ 36	316 $\pm$ 37
	Size (number dist.; nm)	40 $\pm$ 1.8	52 $\pm$ 13	42 $\pm$ 53	24 $\pm$ 10	24 $\pm$ 16	46 $\pm$ 5.8	46 $\pm$ 12
	Zeta potential (mV)	-28 $\pm$ 1.7	-23 $\pm$ 0.3	-17 $\pm$ 8	-0.3 $\pm$ 2.5	2.2 $\pm$ 4.2	-8.6 $\pm$ 1	-8.6 $\pm$ 0.7
TiO <sub>2</sub> NPs	z average (nm)	358 $\pm$ 11	522 $\pm$ 9.3	511 $\pm$ 7.5	830 $\pm$ 105	4234 $\pm$ 321	548 $\pm$ 41	821 $\pm$ 65
	Size (number dist.; nm)	218 $\pm$ 44	224 $\pm$ 62	232 $\pm$ 12	542 $\pm$ 138	1500 $\pm$ 460	234 $\pm$ 12	232 $\pm$ 39
	Zeta potential (mV)	-3.3 $\pm$ 0.3	-20 $\pm$ 0.8	-20 $\pm$ 2.6	0.3 $\pm$ 0.7	-1.1 $\pm$ 0.9	-8 $\pm$ 0.5	-9 $\pm$ 2.1
Ag+TiO <sub>2</sub>	z average (nm)	-	470 $\pm$ 3.4	454 $\pm$ 3.4	641 $\pm$ 17.1	1237 $\pm$ 42	469 $\pm$ 30	671 $\pm$ 148
	Size (number dist.; nm)	-	120 $\pm$ 59	147 $\pm$ 117	369 $\pm$ 185	675 $\pm$ 54	141 $\pm$ 38	132 $\pm$ 63

Zeta potential (mV)	-	-19±0.9	-15±5.4	-1.5±1	-2.2±0.6	-6.2±0.6	-6.1±1.6
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237

238 STEM analysis of Ag NPs and TiO<sub>2</sub> NPs upon co-exposure after 4h of aging in SWW  
239 confirmed the presence of Ti aggregates of approximately 400 nm (Figure 1A) and Ag-rich  
240 nanoparticles of approximately 25 nm and small agglomerates dispersed in clusters (Figure  
241 1B). Moreover, areas with Ag NPs associated with the surface of TiO<sub>2</sub> NPs were also observed  
242 (Figure 1C). Energy-dispersive X-ray spectroscopy (EDS) analysis showed an association of  
243 Ag, Ti, Ca, and P indicating heteroaggregation of Ag NPs with TiO<sub>2</sub> NPs and components of  
244 the wastewater. The elemental distribution suggests the presence of Ca and P at the surface  
245 layers (Figure 1A, C), with the core regions showing heavier mass contrast. A slight increase  
246 in Ag NP z-ave size was observed for pristine Ag NPs (from 75±2.5 to 90±11 nm) in seawater  
247 after 72 h. The larger size of the aged Ag NPs remained in the exposure media (from 268±36  
248 to 316±37 nm), showing the presence of SWW particles in the exposure media. Sp-ICP-MS  
249 showed a size increase of SWW-aged Ag NPs in seawater over time from 28±0.4 nm to  
250 31.4±0.4±1.6 nm (Figure S1). The differences in NP diameter measured with DLS and sp-ICP-  
251 MS are probably due to the higher concentrations needed for DLS that can lead to the aggregate  
252 formation as well as the presence of SWW particles that can influence the signal<sup>9,30</sup>. Therefore,  
253 sp-ICP-MS is a more suitable technique for metallic NP analysis in complex matrices at low  
254 concentrations.



255

256 **Figure 1.** High Angle Annular Dark Field (HAADF) STEM images and elemental analysis of  
 257 Ag and TiO<sub>2</sub> particles (co-incubation) after 4h aging in wastewater. (A) Ti particles (in red)  
 258 associated with Ca (in blue), (B) Ag particles (in green) associated with Ca (in blue) and (C)  
 259 co-localization of Ti (in red) with Ag (in green) and Ca (in blue).

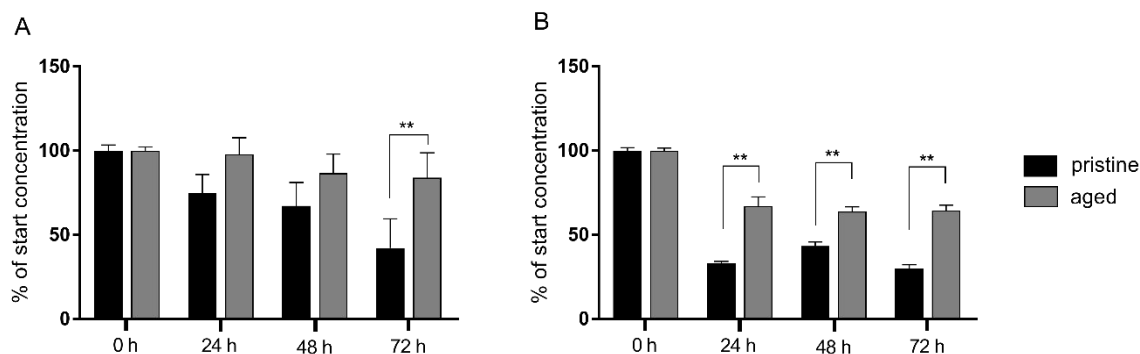
260

261 To determine the influence of SWW particles on the dispersion stability measurements, we  
 262 included measurements of the pure SWW as background controls. The z-ave of SWW (inherent  
 263 particles) in seawater increased from 395±121 nm to 843±499 nm, indicating  
 264 agglomeration/aggregation of SWW particles occurs in seawater. After 72 h, the aggregate size  
 265 and zeta potential of the pristine TiO<sub>2</sub> NPs in seawater were 4234±321 nm and -1.1±0.9 mV,

266 respectively, while they were  $821\pm 65$  nm and  $-9\pm 2.1$  mV, respectively for the aged TiO<sub>2</sub> NPs.  
267 The smaller size of aged TiO<sub>2</sub> NP and the size overlap with particles present in the pure SWW  
268 when added to the exposure media again shows the influence of SWW particles and/or their  
269 interactions with TiO<sub>2</sub> NPs. A similar behavior of smaller aggregate size formation and a  
270 decrease in zeta potential after aging was observed for the mixture of Ag NP and TiO<sub>2</sub> NP in  
271 seawater ( $671\pm 148$  nm). Our results clearly show the influence of SWW particles on size  
272 measurements, which in the case of Ag NP leads to an increased z-ave, and for TiO<sub>2</sub> NP leads  
273 to a smaller z-ave in the exposure media.

274 Further dispersion stability measurements of aged and pristine NP in seawater performed with  
275 UV-vis spectroscopy showed that the effects on particle/aggregate stability are not caused by  
276 SWW particles being present in the size measurements, but are also due to interactions with  
277 the NPs with SWW components. The concentration of Ag and TiO<sub>2</sub> NPs in natural seawater in  
278 the absence of aging decreased over time (Figure 2), indicating either changes in  
279 agglomeration/aggregation state and sedimentation of the formed aggregates or increased  
280 dissolution (for Ag NP). A decrease in UV-vis absorbance of PVP-Ag NPs in seawater (30 ppt)  
281 was attributed to NP dissolution according to a previous study<sup>20</sup>. Aggregation of Ag NPs in  
282 aqueous media of increasing ionic strength has been previously shown<sup>31</sup>. The conductivity (as  
283 an indicator of ionic strength) of the seawater at 35 ppt used in the exposures was 46.7 mS/cm.  
284 This leads to increased aggregation and settling, which could explain the decrease in  
285 concentration and increase in hydrodynamic diameter of Ag and TiO<sub>2</sub> NP over time in  
286 seawater. A higher decrease (60% decrease) in the Ag NP concentration was observed in the  
287 presence of TiO<sub>2</sub> NPs in seawater compared to the single exposures suggesting that TiO<sub>2</sub> NPs  
288 leads to increased Ag NP co-precipitation (Figure S2). In accordance to this study,  
289 heteroaggregation of Ag and TiO<sub>2</sub> NPs at higher TiO<sub>2</sub> NP concentration has been previously  
290 reported<sup>32</sup>. Interestingly, an increase in dispersion stability was observed for both Ag NPs and

291 TiO<sub>2</sub> NPs when the particles were aged for 4 h in SWW, reaching 80% and 60% of the total  
 292 concentration of Ag NPs and TiO<sub>2</sub> NPs, respectively after 72 h in exposure media (seawater)  
 293 (Figure 2).



294

295

296 **Figure 2.** Impact of aging on (A) Ag NP (1 mg/L) and (B) TiO<sub>2</sub> NP (10 mg/L) dispersion  
 297 stability in natural seawater over time. The concentration of pristine (freshly prepared, black  
 298 bars) or aged (4 h in SWW, grey bars) NP in natural seawater was determined with UV-Vis at  
 299 0, 24, 48 and 72 h. The data is presented as mean  $\pm$  SD. Asterisks denote statistically significant  
 300 differences ( $p < 0.01$ ).

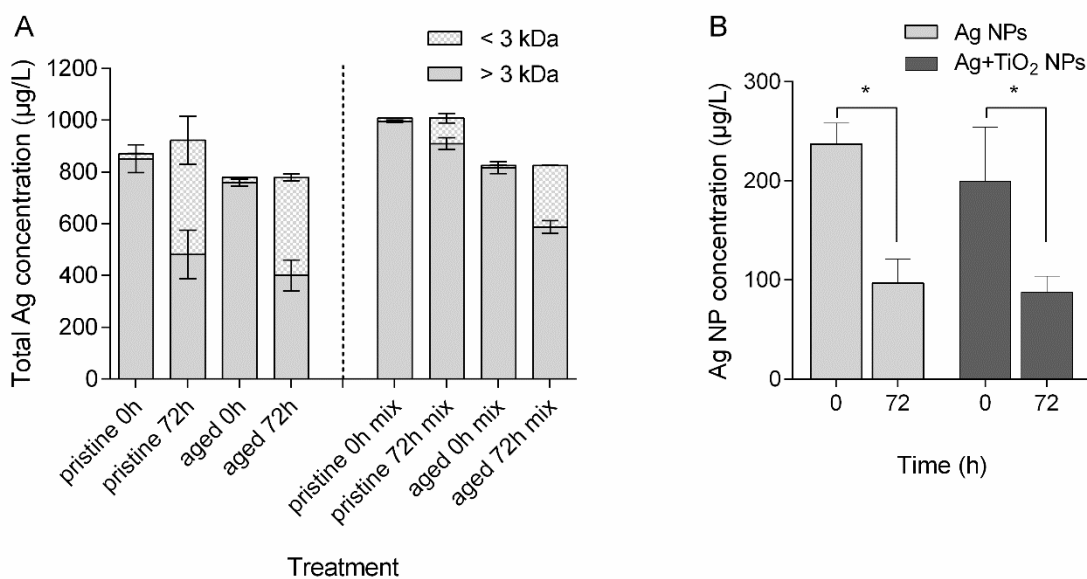
301

302 Previous studies have demonstrated an increased dispersion stability of Ag NPs in moderately  
 303 hard reconstituted water containing humic acids compared to media with higher chloride (Cl<sup>-</sup>)  
 304 concentrations<sup>21</sup>. Moreover, it has been recently shown that TiO<sub>2</sub> NPs were more stable over  
 305 time in natural water samples containing high molecular weight biopolymers<sup>33</sup> and in seawater  
 306 in the presence of dissolved organic carbon despite the high salinity levels of 30 ppt<sup>34</sup>. This  
 307 could explain the increased dispersion stability observed in the case of the Ag NP aged in SWW  
 308 that contains high levels of total organic carbon and proteins. In the current study natural



309 organic matter was not included in the synthetic wastewater which could lead to further  
310 increase in stability depending on the natural organic matter quality and media ion  
311 composition<sup>8</sup>.

312 **Total, dissolved and particulate Ag.** At the highest nominal exposure concentration of 1  
313 mg/L, the Ag NP dissolution was observed to increase over time, reaching measured  
314 concentrations of 441  $\mu\text{g/L}$  and 378  $\mu\text{g/L}$ , respectively for pristine and aged NP after 72h in  
315 seawater (Figure 3A). This represents dissolution of  $48\pm 10\%$  and  $49\pm 2.8\%$  of the total Ag  
316 added (1 mg/L) for pristine and aged, respectively. A low dissolution rate of approximately 4%  
317 has previously been reported for Ag NPs (NM 300K, EU JRC Repository) in both bacterial  
318 media and artificial wastewater<sup>35</sup>. The lower dissolution reported is probably due to the shorter  
319 experimental time of 1 h (compared to 72 h in the current study), as well as the higher  
320 concentration used in the dissolution experiment (50 mg/L compared to 1 mg/L used in the  
321 current study). The dissolution rate has previously been shown to be affected by both the  
322 concentration and size of NPs, with a decrease in aggregate size and an increase in dissolution  
323 as the NP concentration decreases<sup>19-21</sup>. Moreover, it has been previously shown that the Cl/Ag  
324 ratio determines the dissolution rate with increasing Cl/Ag ratio leading to increased  
325 dissolution and increased presence of  $\text{AgCl}_x^{(x-1)-}$  soluble species compared to AgCl (s). In the  
326 current study, the Cl/Ag ratio is 19400 and so the formation of soluble Ag-Cl complexes is  
327 expected<sup>36</sup>. Despite the observed increased stability during aging measured with UV-vis the  
328 soluble Ag levels measured with ICP-MS were similar for both aged and pristine particles in  
329 seawater. This discrepancy could result from interference in UV-vis absorbance due to  
330 aggregation<sup>20,21</sup>, sorption of constituents present in the media<sup>20</sup> and the presence of particles in  
331 the SWW. It has been previously discussed that there is no ideal technique for the  
332 characterization and detection of NPs in complex environmental matrices but rather a  
333 combination of multiple analytical techniques is needed<sup>30</sup>.



334

335 **Figure 3.** (A) Total and dissolved measured Ag concentrations of pristine and aged (in SWW)  
 336 Ag NPs (nominally 1 mg/L) over time in seawater, either as single or combined exposures with  
 337 TiO<sub>2</sub> NPs (nominally 10 mg/L). (B) Measured NP concentration of aged Ag NPs (nominally 1  
 338 mg/L) in single exposures and in combination with TiO<sub>2</sub> NPs as measured over time using sp-  
 339 ICP-MS. The total, dissolved Ag (centrifugal ultrafiltration using a 3kDa filter membrane-ICP-  
 340 MS) and particulate form (sp-ICP-MS) were determined after 0 h and 72 h of exposure. The  
 341 values shown are measured concentrations. The error bars represent the standard deviation of  
 342 three replicates. Asterisks denote statistically significant differences ( $p < 0.05$ ).

343

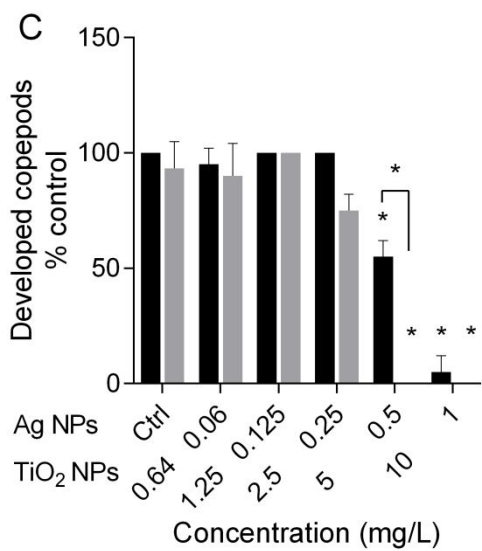
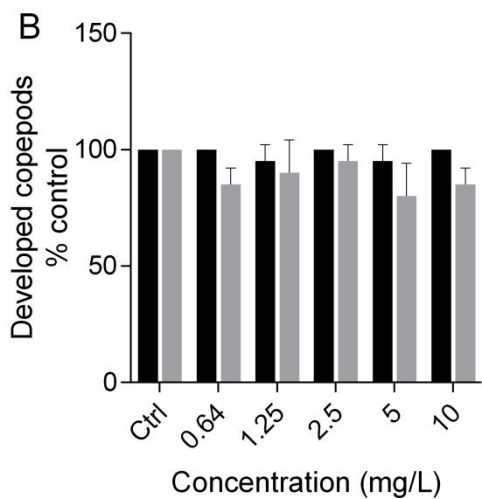
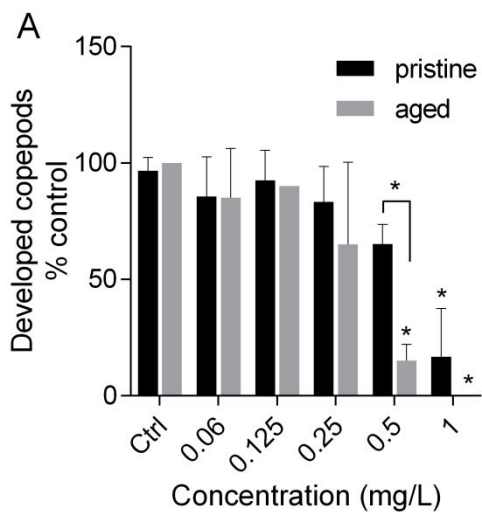
344 Relative to the Ag NP single exposures, a decrease in the dissolved Ag concentration was  
 345 measured in samples containing the Ag NP and TiO<sub>2</sub> NP mixture in both pristine and aged  
 346 exposures (Figure 3A). This indicates that the presence of TiO<sub>2</sub> either inhibits the dissolution  
 347 process or that the dissolved species rapidly become associated with surface of the TiO<sub>2</sub> NPs.  
 348 In accordance with the current findings, a previous study has shown that under illumination,  
 349 TiO<sub>2</sub> NPs led to a 64.3% decrease in Ag<sup>+</sup> release from Ag NPs compared to Ag NP only

350 exposures and the reformation of Ag NPs on the surface of TiO<sub>2</sub> NPs<sup>22</sup>. Similarly, lower Ag<sup>+</sup>  
351 levels were measured when Ag NPs and TiO<sub>2</sub> NPs were present as a mixture in lake water,  
352 where an increase in Ag<sup>+</sup> sorption to TiO<sub>2</sub> particles was observed<sup>23</sup>. It should be noted that the  
353 presence of Ag-containing solids that do not pass through the 3kDa membrane could be omitted  
354 from the analysis leading to an underestimation of the dissolved Ag species.

355 The behavior of Ag NPs during exposures of SWW-aged and pristine particles was also studied  
356 with sp-ICP-MS, a technique that has low detection limits and can provide size and particle  
357 concentration at environmentally relevant concentrations. In the exposure media, a decrease in  
358 the Ag NP measured concentration from 237 µg/L to 96.9 µg/L (Figure 3B) was observed after  
359 72h exposure (exposure media renewal), which is in accordance with the increased dissolution  
360 with time. Similar results using sp-ICP-MS analysis have been previously reported, showing  
361 increased Ag dissolution over time accompanied by a decrease in particle number that indicates  
362 transformation to dissolved or complexed Ag or formation of smaller-sized Ag NPs<sup>37</sup>.  
363 Moreover, secondary particles were shown to form during Ag NP dissolution in complex  
364 matrices such as wastewater effluent samples that contain high concentrations of amino acids<sup>38</sup>.  
365 The lower soluble Ag levels observed in the presence of TiO<sub>2</sub> in the current study, together  
366 with the lower Ag NP concentration measured with sp-ICP-MS, indicate a reformation of Ag  
367 particulate complexes with TiO<sub>2</sub> after dissolution or formation of smaller sized particles. Due  
368 to the limit of detection of sp-ICP-MS, particles below 19 nm will be incorporated into the  
369 dissolved fraction, therefore the presence of smaller-sized Ag complexes are not measured.

370 **Effects of particles on *T. battagliai* naupliar development.** A developmental rate of 0.28 d<sup>-1</sup>  
371 was determined in both natural seawater and SWW (10% SWW in natural seawater) controls  
372 and this value was set to represent 100%. An increased toxicity was observed upon exposure  
373 to the positive AgNO<sub>3</sub> control, resulting in an EC<sub>50</sub> of 9.86 µg/L (SI, Figure S2), which is in

374 the same range as in previous studies with the copepod *Amphiascus tenuiremis*<sup>39</sup> and confirms  
375 the increased sensitivity of the naupliar developmental stage. Exposure to increasing  
376 concentrations of Ag NPs led to delayed development at concentrations above 500 µg/L with  
377 a complete cessation of molting and increased mortality (Figure 4A). The aging process led to  
378 increased toxicity compared to the pristine Ag NPs with a 2-fold decrease in EC50 (316 µg/L)  
379 compared to pristine Ag NPs in natural seawater (EC50 = 640 µg/L). At the highest exposure  
380 concentration of 1 mg/L the measured dissolved Ag concentrations were at similar levels for  
381 both aged and pristine exposures (441 µg/L and 378 µg/L, respectively), suggesting that the  
382 effects and toxicity cannot be predicted by the dissolved or total Ag concentrations but the  
383 exposure media composition and complexity should also be taken into account. Ag NP aging  
384 in the complex SWW media containing high levels of organic matter could lead to increased  
385 residence times in the water column and increased exposure. It has been previously shown that  
386 Ag NPs spiked in crude wastewater resulted in lower toxicity to the bioluminescent bacteria  
387 *Pseudomonas putida* compared to spiked final wastewater<sup>28</sup>. The differences in toxicity  
388 observed despite the similar Ag levels measured can be due to differences in interaction  
389 between dissolved Ag and Ag ions/NPs attached to SWW particles during aging with different  
390 tissues/external surfaces of the organism.



391

392 **Figure 4.** Effects of pristine vs aged NPs on the naupliar development of *T. battagliai*. The  
393 nauplii were exposed for 6 days to (A) Ag NPs (0-1 mg/L), (B) TiO<sub>2</sub> NPs (0-10 mg/L) and (C)  
394 a mixture of Ag and TiO<sub>2</sub> NPs at increasing concentrations. The exposure media were renewed  
395 after 72 h. The values shown are nominal exposure concentrations.

396 No effects on *T. battagliai* development rate or viability were observed upon exposure to  
397 TiO<sub>2</sub> NPs despite the high exposure concentrations employed (0.64-10 mg/L) and irrespective  
398 of the aging process (Figure 4B). Similar results have also been recently observed for  
399 rainbow trout exposed to either wastewater-borne or water dispersed TiO<sub>2</sub> NPs where no  
400 effects on mortality, behavior and growth were found<sup>16</sup>.

401 A similar pattern to that observed for the Ag NP only exposures was also observed when the  
402 nauplii were exposed to the mixture of aged Ag and TiO<sub>2</sub> NPs (Figure 4C), with a dose-  
403 dependent effect on naupliar development and number of organisms reaching the copepodid  
404 stage leading to an EC50 of 270 µg/L. In the combined exposures, higher dissolved Ag levels  
405 were measured for the aged Ag NPs (238.3 µg/L) compared to the pristine Ag NPs (98.57  
406 µg/L) (Figure 3A). Increased release of Ag ions in the presence of TiO<sub>2</sub> NPs was previously  
407 described under sunlight irradiation with the simultaneous formation of Ag<sup>0</sup> particles of smaller  
408 size<sup>32</sup>. The increased toxicity may be due to one or a combination of increased dissolution, re-  
409 formation of smaller Ag particles and the presence of TiO<sub>2</sub> particles that combined lead to  
410 enhanced effects through a different mode of action. In a previous study, TiO<sub>2</sub> led to enhanced  
411 Ag NP toxicity to *Tetrahymena pyriformis* under illumination conditions, which was associated  
412 with oxidative stress and a formation of Ag-TiO<sub>2</sub> particle complexes after Ag<sup>+</sup> sorption to the  
413 surface of TiO<sub>2</sub> particles<sup>22</sup>. Increased Ag toxicity to *Daphnia magna* has also been previously  
414 observed in the presence of TiO<sub>2</sub> with higher Ag body burdens that were explained by ingestion  
415 of Ag-sorbed TiO<sub>2</sub> NPs<sup>40</sup>. In the current study, the observed differences in toxicity are

416 suggested to be due to the dynamic nature of the exposures, where organisms are being exposed  
417 to (i) a mixture of both particles and dissolved species released over time and (ii) different  
418 particulate complexes formed over time, which is in agreement with previous studies<sup>39</sup>. It  
419 should be noted that the NP behavior and subsequent exposure to *T. battagliai* may be altered  
420 by the presence of the algae. NP agglomeration/interaction with released extracellular  
421 polymeric substances and deposition can alter the actual exposure, while NP/ion interaction  
422 with the algae surface during the exposures can also lead to decrease in the dissolved species  
423 measured<sup>39</sup>. Moreover, the presence of food in the exposure system can lead to increased  
424 bioavailability due to Ag ions/NPs with the food source and increased uptake<sup>41</sup>.

425 **Ag NP uptake determination with sp-ICP-MS.** The Ag NP concentration retained within *T.*  
426 *battagliai* after 6 day exposure and a 24 h depuration period was evaluated with sp-ICP-MS in  
427 organisms exposed to concentrations at which no developmental effects were observed; 125  
428  $\mu\text{g/L}$  pristine Ag NPs, 125  $\mu\text{g/L}$  aged Ag NPs, mixture of 125  $\mu\text{g/L}$  Ag and 1250  $\mu\text{g/L}$   $\text{TiO}_2$   
429 NPs (aged), 6  $\mu\text{g/L}$   $\text{AgNO}_3$  (nominal concentrations). Ag particles in the nanoscale were  
430 detected in *T. battagliai* in all treatments, with an average size of 25 nm in treatments  
431 containing Ag NPs (alone or combined with  $\text{TiO}_2$  NPs) and 40 nm in the  $\text{AgNO}_3$  treatment  
432 (Table 2). Among the particle exposures, the highest Ag concentration retained in the  
433 organisms after 24 h depuration was found in the groups exposed to the pristine Ag NPs, where  
434 3.45% of Ag NPs were retained (0.30  $\mu\text{g}/\text{copepod}$ ) relative to the Ag NP levels measured in  
435 the exposure media (8.83  $\mu\text{g}$  Ag NPs/L). Lower Ag NPs levels were measured in organisms  
436 exposed to the aged Ag NPs, with a 2.4 times lower Ag NP percentage retention compared to  
437 the pristine Ag NPs, reaching 0.08  $\mu\text{g}$  Ag NPs/L (1.44% retention) and indicating a decreased  
438 bioavailability of the aged particles compared to the pristine counterparts. These results are in  
439 accordance with previous studies showing that Ag bioavailability to the alga *Chlamydomonas*  
440 *reinhardtii* is decreased in wastewaters compared to simplified media<sup>37</sup>. Similarly, a limited

441 bioavailability of wastewater-borne Ag NPs to rainbow trout upon aqueous exposure compared  
 442 to the pristine counterparts has been reported presumably due to particle transformation<sup>42</sup>.  
 443 Despite the lower bioavailability, the complexed species and smaller Ag NP particles being  
 444 formed in wastewater media can still be taken up by the copepods.

445

446 **Table 2.** Overview of Ag NP content and size measurement with sp-ICP-MS in exposure  
 447 media and *T. battagliai* (measured concentrations) after 24 h of depuration, with and without  
 448 NP aging.

Exposure	Exposure media			<i>T. battagliai</i>		
	Size (nm)	Measured conc ( $\mu\text{g Ag NPs/L}$ )	Size (nm)	Measured conc ( $\mu\text{g Ag NPs/copepod}$ )	% Ag NPs retained in organisms	
Pristine	Ag	27.17	8.83 $\pm$ 3.2	24.09	0.30 $\pm$ 0.13	3.45
	AgNO <sub>3</sub>	40.18	1.99 $\pm$ 0.13	38.35	0.23 $\pm$ 0.19	11.74
Aged	Ag	25.53	5.83 $\pm$ 1.48	25.11	0.08	1.44
	Ag +TiO <sub>2</sub>	24.53	5.23 $\pm$ 1.03	19.08	0.15	2.82

449

450 In the presence of TiO<sub>2</sub>, a higher concentration of Ag NPs was retained in the organisms  
 451 compared to the Ag NP single exposure (~2 times higher percentage Ag NP retention). A higher  
 452 bioavailability for aqueous Ag compared to Ag in particulate form has been previously reported  
 453 for uptake studies using the algae *C. vulgaris*<sup>43</sup>. Similarly, the highest Ag levels retained in the  
 454 organisms (11.7%) relative to the measured Ag levels in the exposure media were observed in  
 455 the AgNO<sub>3</sub> treatment indicating an increased bioavailability which is in accordance with  
 456 previous studies reporting higher Ag uptake in *Daphnia magna* in aqueous exposures of  
 457 AgNO<sub>3</sub> compared to Ag NPs<sup>44</sup>. An average particle size of ~40 nm was measured in the  
 458 organisms exposed to AgNO<sub>3</sub>, which suggests a particle formation within the organisms after



459 exposure to the ionic form. A biogenic formation of Ag NP forms from ionic Ag has been  
460 previously detected in earthworms *Lumbricus rubellus* and *Eisenia fetida* tissues exposed to  
461  $\text{AgNO}_3$ <sup>45,46</sup>, and it was suggested this represented a protection and detoxification mechanism.  
462 The formation of granules containing metals, including Ag, with sulfur or phosphorus  
463 precipitates within cell vacuoles has been shown to play an important role in metal  
464 sequestration and detoxification process in invertebrates including crustaceans<sup>47</sup>. The use of  
465 sp-ICP-MS in combination with an enzymatic digestion method allowed the detection of Ag  
466 NPs in complex matrices such as whole organisms at low concentrations and helped towards  
467 gaining an improved understanding of particle behavior in complex matrices and interpreting  
468 effects data.

469 **Environmental Implications.** The continuous NP release and enhanced toxicity that can be  
470 observed in co-exposure with other NPs and the NP altered behavior in different environmental  
471 media can lead to toxicity that cannot be explained or predicted based on total, soluble or  
472 particulate concentrations. The nature of the NP behavior and association with organic  
473 matter/biomolecules and the increased sensitivity observed for (epi)benthic organisms raise  
474 concerns over the environmental impact of settling NP in natural systems. Importantly, the  
475 complexity and environmental relevance of the matrix selected for use in exposure experiments  
476 can have a significant impact on the dispersion stability, agglomeration/aggregation and  
477 dissolution behavior of NPs and their subsequent effects on exposed organisms. The  
478 concentrations used in the current study were higher than the ones currently found in the  
479 environment, therefore future research efforts should focus on more environmentally relevant  
480 aging conditions at environmentally relevant concentrations and in combination with other  
481 environmental stressors including co-contaminants and elucidate factors that lead to altered NP  
482 bioavailability.

## 483 ASSOCIATED CONTENT

484 **Supporting Information.** Additional information is provided on synthetic wastewater  
485 composition, NP characterization methodology (STEM/EDS, sp-ICP-MS), the size  
486 distribution of Ag NPs during aging and exposures obtained by sp-ICP-MS (Figure S1), UV-  
487 vis measurements of pristine and aged Ag and TiO<sub>2</sub> NPs in combined exposures over time  
488 (Figure S2), effect of AgNO<sub>3</sub> on *T. battagliai* naupliar development (Figure S3), Ag NP  
489 characterization (size and concentration) during aging in SWW and exposure in SW over  
490 time in single and combined exposures with TiO<sub>2</sub> NPs obtained by sp-ICP-MS (Table S1),  
491 measured total Ti concentration of pristine and aged TiO<sub>2</sub> NPs in single and combined  
492 exposures with Ag NPs at the start of exposures (Table S2).

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497 **Author Contributions**

498 The manuscript was written through contributions of all authors. All authors have given  
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500

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