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Transfer and Ecotoxicity of Titanium Dioxide Nanoparticles in the Terrestrial and Aquatic Ecosystems: A Microcosm Study

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1 **Transfer and Ecotoxicity of Titanium Dioxide Nanoparticles in Terrestrial and Aquatic**2 **Ecosystems: A Microcosm Study**3 Vinita Vijayaraj,[†] Clarisse Liné,[†] Stéphanie Cadarsi,[†] Clément Salvagnac,[†] David Baqué,[†]
4 Arnaud Elger,[†] Maialen Barret,[†] Florence Mouchet,[†] and Camille Larue^{*,†}

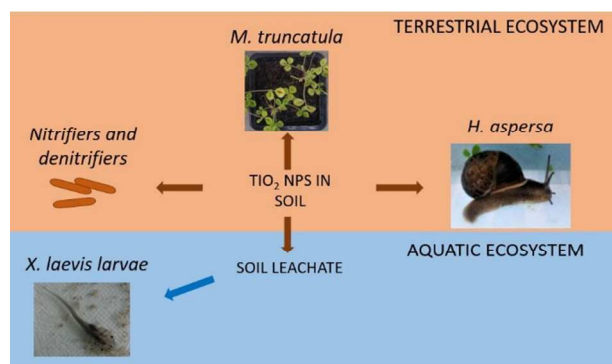
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10 **Graphical abstract**

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12

13 **ABSTRACT:** With the advancement in nanotechnology, particularly the use of TiO₂
14 nanoparticles (NPs), there is a need to study their release into the environment and assess the
15 related risk in an environmentally relevant contamination scenario. In the present study, the
16 transfer and toxicity of TiO₂ NPs in microcosms mimicking a terrestrial and an aquatic
17 ecosystems were evaluated. The contaminated soil was prepared by spiking natural soils and
18 used as the basis for all exposure systems including preparation of soil leachates for amphibian
19 exposure. Results demonstrated significant reductions in bacterial (-45%) and archaeal (-36%)
20 nitrifier abundance; significant translocation of Ti to *M. truncatula* leaves (+422%); significant
21 reductions in plant height (-17%), number of leaves (-29%), and aboveground biomass (-53%);
22 non-significant Ti uptake in snail foot and viscera, and excretion in feces; and genotoxicity to *X.*
23 *laevis* larvae (+119% micronuclei). Our study highlights a possible risk of engineered TiO₂ NPs
24 in the environment in terms of trophic transfer and toxicity in both terrestrial and aquatic
25 environments.

26 INTRODUCTION

27 Nanoparticles (NPs) are particles with at least one dimension smaller than 100 nm.¹ Compared
28 with their micro/macro particle counterparts, NPs have a larger surface area over volume ratio,²
29 and number of surface atoms, which results in increased surface energy and reactivity.³ The
30 understanding of their unique properties has rendered them indispensable in several fields of
31 science and technology. Although not all NPs may be toxic, because they are manufactured in a
32 variety of sizes and shapes for use in a multitude of products and industrial processes,⁴ they
33 could pose different potential hazards.^{5,6} Despite the recommendation by the National Organic
34 Standards Board in 2010, products containing NPs are still being manufactured worldwide.⁴ It is
35 therefore necessary to understand the risks associated with their release in the environment.

36 Among the most widely used NPs are silver and titanium dioxide (TiO₂) NPs.⁷ TiO₂ NPs are
37 commonly known for their photocatalytic applications and used as semiconductors,⁸ additives in
38 food,⁹ and in cosmetic products.¹⁰ Ti is also a widespread element in the environment, with
39 concentrations in soils ranging for instance from 43 mg kg⁻¹ in sand to 1671 mg kg⁻¹ in clayey
40 soil.¹¹ The concern, however, is the increase in this background concentration beyond an
41 environmentally safe threshold, in addition to the potential toxicity of these NPs, on their own,
42 due to the differences in their properties and behavior when compared with natural Ti.
43 Ultimately these NPs will reach the environment.¹² In wastewater treatment plants, NPs partition
44 between treated water and sludge effluents. Through the discharge of treated water, NPs can
45 reach aquatic ecosystems. But the majority (99%)¹³ that remains in sewage sludge is dispersed
46 into agricultural fields as fertilizer.¹⁴ Runoff from these polluted soils can in turn reach water
47 bodies. A recent study by Sun *et al.*¹⁵ predicted the concentrations of TiO₂ NPs in surface waters
48 to be ~ 2.17 µg L⁻¹, in sludge 1.60 g kg⁻¹, and in sludge-treated soils 61.10 mg kg⁻¹. An

49 accumulation in both terrestrial and aquatic organisms could be carried upward into the food
50 chain by primary and secondary consumption routes.

51 Negative effects have already been noticed, as a result of NP exposure, in terrestrial and aquatic
52 organisms at the single-organism scale,¹⁶ but the study of NP effects in a simplified food web is
53 not very common. Suggestions have been made that certain NPs can bioaccumulate and possibly
54 bio-magnify in the trophic chain.^{17,18} Yeo and Nam¹⁹ compared the bioaccumulation of TiO₂ NPs
55 and carbon nanotubes in a paddy ecosystem, and observed a bioaccumulation from plankton to
56 ricefish. In a microcosm containing soil, lettuce, crickets, and lizards, Servin *et al.*²⁰
57 demonstrated that the availability of CuO NPs to terrestrial organisms can be modified by
58 weathering. Using leaf litter decomposition as a functional marker for ecotoxicity in freshwater
59 mesocosms, Bour *et al.*²¹ showed that this ecosystem function was not impacted by CeO₂ NP
60 exposure, in line with the absence of significant differences in numbers, growth, or development
61 of chironomid larvae. However, studies are lacking in terms of a real-life scenario where transfer
62 of TiO₂ NPs occurs from the soil into the water compartment and their impact on, and uptake by,
63 relevant organisms of these environments.

64 In the present study, the aim was to combine two microcosms mimicking both terrestrial (soil,
65 soil microbial communities, alfalfa plants, and snails) and aquatic (soil leachates and amphibian
66 larvae) compartments to (a) determine whether there is a transfer of Ti within and between these
67 two compartments and (b) evaluate the toxicity of TiO₂ NPs to soil microbial communities (total
68 abundance and abundance of functional guilds), plant (height, number of leaves, aboveground
69 biomass, chlorophyll), snail (mortality, avoidance behavior), and amphibian larvae (mortality,
70 growth, and genotoxicity). Our study contributes valuable data in terms of the impact of TiO₂

71 NPs on environmentally relevant bioindicators of pollution and provides an insight into
72 implications for food safety and ecotoxicological risk assessment.

73

74 1. MATERIALS AND METHODS

75 1.1. TiO₂ NP characterization and suspension preparation

76 TiO₂ NPs used in this study were provided by Sigma-Aldrich (P25); they have been widely used
77 in ecotoxicological studies, permitting easy comparisons. They consist of 80% anatase and 20%
78 rutile with a nominal diameter of 25.0 ± 5.7 nm; are polyhedral, uncoated, with a specific surface
79 area of 46 ± 1 m² g⁻¹ and zeta potential in ultrapure water of -10.5 ± 2.6 mV (File S1).²² The
80 TiO₂ NP suspension was freshly prepared before soil contamination (10000 mg L⁻¹); it was
81 vortexed for 30 s and agitated while pipetting to keep it homogenized.

82

83 1.2. Terrestrial exposure

84 1.2.1. Exposure medium preparation

85 The two main conditions in this study were the control soil and the spiked soil, from which all
86 conditions were derived. Soils were silty sand (Lufa Speyer 2.1; File S2) prepared with 20%
87 (w/w) commercial compost to sustain proper plant growth (final soil mixture pH: 5.3, organic
88 matter content: 12.6%). For the spiked soil, 500 mg kg⁻¹ of TiO₂ NPs were added to mimic
89 several years of sewage sludge spreading on agricultural soils.¹⁵ Both control and spiked soils
90 were mixed with either Millipore water or with NP suspension at a ratio of 1: 1 (w:w). After
91 shaking at 100 rpm for 2 h, soils were filtered to eliminate excess water, dried, and transferred to

92 small plastic pots, 7 × 7 × 6.4 cm (Soparco, France). This mixing procedure was chosen to
93 ensure a homogenous contamination of the soil matrix.

94

95 1.2.2. *Medicago truncatula* exposure to TiO₂ NPs

96 *Medicago truncatula* (ecotype A17) is a model for legume plants,²³ chosen for their implication
97 in the nitrogen cycle.²⁴ Seeds were kindly provided by Dr. Manuel Gonzalez-Guerrero,
98 Universidad Politécnica de Madrid, Spain. They were first scarified and soaked overnight in
99 water, in the dark. They were then placed in Petri dishes (6 to 7 seeds/Petri dish) with Hoagland
100 agar medium for germination at 4°C for 72 h, before transferring to the soil pots. Seedlings (*n* =
101 10 per condition) were biomonitored every day for the number of leaves, height, and chlorophyll
102 levels using a SPAD-meter (SPAD-502 chlorophyll meter, Minolta Camera Co., Japan). After 60
103 days, plants were harvested. Dry weights were recorded after 48 h at 60°C prior to Ti
104 quantification in leaves.

105

106 1.2.3. Impact of TiO₂ NPs on soil microbial communities

107 The microbiological analysis focused on soil nitrifiers and denitrifiers to further investigate NP
108 impact on the nitrogen cycle. Upon plant harvest, three cores of whole-depth bulk soil, deprived
109 of roots (and without further processing), were collected at 3 cm from the stem axis from
110 individual soil pots (*n* = 6 per condition). The three cores were homogenized, and 300 mg
111 composite subsamples were stored at -20°C until DNA isolation. The FastDNA SPIN Kit for
112 Soil was used according to the manufacturer's instructions (MP Biomedicals laboratories, Unites
113 States). The quality and quantity of DNA extracts were determined using a NanoDrop
114 spectrophotometer. Quantitative PCR (qPCR) was used to measure the abundance of total soil

115 bacteria targeting the universal 16S rRNA gene as well as archaeal ammonia monooxygenase A
116 genes (*amoA*) for ammonia oxidizing archaea (AOA, nitrifiers), bacterial *amoA* for ammonia
117 oxidizing bacteria (AOB, nitrifiers), and the bacterial nitrite reductase (*nirK* and *nirS*) and the
118 nitrous oxide reductase (*nosZ*) genes for denitrifiers.²⁵ For more details about the experimental
119 procedure, see File S3.

120

121 1.2.4. *Helix aspersa* exposure to TiO₂ NPs

122 Snails were chosen for this experiment as sensitive bioindicators of pollution in the
123 environment.²⁶ They were obtained from a snail breeding farm to ensure homogeneity. After a 48
124 h depuration step, they were transferred to clean boxes containing 5 g of dry soil (after plant
125 harvest) mixed with 3 mL of Millipore water (*n* = 6 per condition). Soil moisture was set high
126 (189% of the water holding capacity), to sustain high hygrometry in microcosms and thus snail
127 activity. The experimental model included only soil exposure, which was renewed each day for
128 10 days; snail feces were collected daily. After removal, soils were dried and weighted to
129 determine the quantity consumed by each snail over 24 h. Following 72 h of depuration, snails
130 were rinsed and stored at -20°C. Snail foot and viscera were separated and dried prior to Ti
131 quantification.²⁶

132

133 1.3. Aquatic exposure

134 1.3.1. Exposure media for amphibian larvae

135 Exposure media for amphibian larvae were prepared using a new batch of control and spiked
136 soils. Soils (1 kg of dry matter) were mixed with 10 volumes (ratio 1:10) of mineral water
137 (Volvic®; Ti under detection limit of inductively coupled plasma–optical emission spectroscopy

138 [ICP-OES]) according to the adapted static leaching procedure of the French norm XP 31-210
 139 (AFNOR, 1992) without filtration.²⁹ After 24 h of stirring, the mixtures were allowed to settle for
 140 2 h, and the supernatant suspensions were collected by siphoning into glass flasks. Ten different
 141 exposure conditions were set up (Table 1).

142

143 Table 1: Amphibian larvae exposure conditions (CS : control soil, SS : spiked soil)

Condition	Preparation
NC	Pure Volvic® water
PC	40 mg L ⁻¹ cyclophosphamide in Volvic® water
ICP equiv of CS	3.6 mg L ⁻¹ TiO ₂ NPs in Volvic® water
CS Raw	Raw leachates from control soil
CS50	50% raw leachates from control soil + 50% Volvic® water
CS25	25% raw leachates from control soil + 75% Volvic® water
ICP equiv SS	12.3 mg L ⁻¹ TiO ₂ NPs in Volvic® water
SS Raw	Raw leachates from spiked soil
SS50	50% raw leachates from spiked soil + 50% Volvic® water
SS25	25% raw leachates from spiked soil + 75% Volvic® water

NC = negative control; PC = positive control (genotoxicity assay); ICP equiv of CS = equivalent Ti concentration of CS in Volvic®; ICP equiv SS = ICP equivalent Ti concentration of SS in Volvic®.

144

145 1.3.2. Impact of TiO₂ NPs on *Xenopus laevis*

146 *X. laevis*, a widely used model in ecotoxicology,^{27,28} are filter-feeders, and can be contaminated
147 via skin contact, water filtration *via* gills, and breeding exposure. *X. laevis* larvae (stage 50 of the
148 development table of Nieuwkoop & Faber³⁰) were exposed in semi-static conditions based on
149 modified ISO 21427-1.³¹ The larvae ($n = 15$ per condition) were taken from the same hatch to
150 reduce inter-animal variability. They were submitted to daily renewal of media and fed with fish
151 food TetraPhyll®. After 12 days of exposure, mortality was recorded and larvae anaesthetized
152 (MS222, Sandoz, France) to take pictures and evaluate growth inhibition (Image J). Genotoxicity
153 was assessed through the micronucleus test³¹: blood was extracted from the heart of each
154 anesthetized larva, and micronucleated erythrocytes per thousand cells (ME‰) were counted
155 under optical microscope following hematoxylin-eosin staining.

156

157 1.4. Ti uptake: acid digestion and ICP-OES

158 Acid digestion was done in a DigiPrep® heating system, using HNO₃, NH₄F, and HClO₄ as well
159 as H₂O₂, with a temperature of 100°C and several evaporation steps.¹¹ Digested samples were
160 diluted in 5% HNO₃. For soil, a filtration step was necessary (0.45 µm syringe filters). The
161 samples were then analyzed by ICP-OES with control samples (blanks with only chemicals and
162 standard reference material NIST 1573a: tomato leaves) (IRIS Intrepid II XDL, Thermo
163 Scientific, United States). Ti recovery from TiO₂ NPs has been calculated to be $88.2 \pm 2.1\%$.¹¹

164

165 1.5. Statistical Analysis

166 Data were checked for homoscedacity and normality. When assumptions were met for
167 parametric analyses, a Welsh *t*-test or a one way ANOVA were used. Otherwise, a Wilcoxon test

168 or a Kruskal-Wallis test followed by a Dunn's post hoc test were applied. A principal component
169 analysis (PCA) was carried out on plant elemental content to investigate TiO₂ NP impact on plant
170 nutrition. All statistical analyses were performed using the R statistical software (version
171 3.1.3),³² with `car`,³³ `multcompView`,³⁴ `pgirmess`,³⁵ and `lsmeans`³⁶ packages (all results are
172 gathered in Table S4). Data is displayed as median ± interquartile range, average is shown by a
173 red dot.

174

175 2. RESULTS AND DISCUSSION

176 2.1. Terrestrial ecosystem

177 2.1.1. Ti concentration in soil

178 As expected, the addition of TiO₂ NPs lead to a significant increase in Ti concentration in spiked
179 soil in comparison with the control soil ($p = 0.01$). The average concentration in the control soil
180 was $278.0 \pm 13.4 \text{ mg kg}^{-1}$, while that in spiked soil was $341.7 \pm 12.2 \text{ mg kg}^{-1}$ (see File S5A).

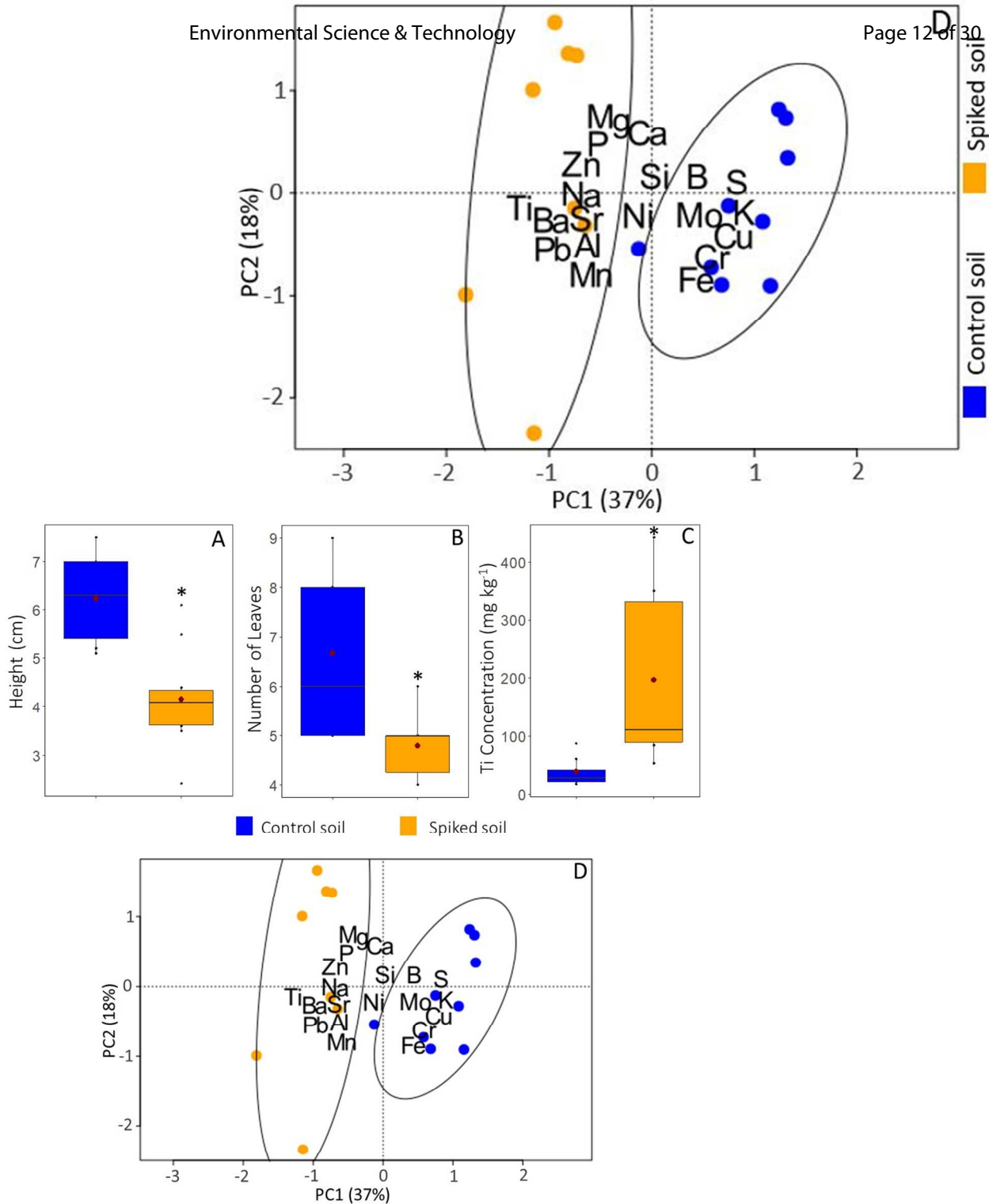
181 Although the soils were spiked with 500 mg kg^{-1} TiO₂ NPs ($300 \text{ mg Ti kg}^{-1}$ equivalent), the
182 average increased concentration of Ti in spiked soils after leaching was below 300 mg kg^{-1} Ti.
183 Considering that the soil used in this study was 88% sandy, this could be explained as a loss of
184 Ti into the water compartment during the leaching procedure, as suggested by our previous
185 results.¹¹

186

187 2.1.2. TiO₂ NP phytotoxicity and uptake by *Medicago truncatula*

188 After 60 days of exposure, plants grown in the spiked soil were significantly smaller than those
189 grown in the control soil ($p < 0.001$) with average heights of $4.3 \pm 0.3 \text{ cm}$ and $6.2 \pm 0.3 \text{ cm}$,

190 respectively (Figure 1A). Plant heights were similar until day 12, after which exposed plants had
191 a 17% smaller size (File S5B). Along the development, non-exposed plants produced
192 significantly more leaves ($p = 0.01$) with *ca.* seven against five leaves for exposed plants (Figure
193 1B). A significant reduction in aboveground biomass (-53%) was also observed for exposed
194 compared to non-exposed plants ($p < 0.001$; File S5C). Chlorophyll levels tended to be lower in
195 the spiked conditions than in the control conditions, but no significant difference was detected
196 (File S5D). In terms of Ti uptake, concentration in the exposed leaves was significantly higher
197 compared to the non-exposed leaves ($p < 0.01$) with respective concentrations of 196.6 ± 53.1
198 mg kg^{-1} and $37.6 \pm 7.9 \text{ mg kg}^{-1}$ (Figure 1C). Exposure to TiO_2 NPs tended to impact plant
199 ionome: exposed plants were significantly enriched in zinc, lead, sodium, manganese,
200 phosphorus but contained less copper, iron, potassium, sulfur, molybdenum, and boron (Figure
201 1D).



202

203 Figure 1: A: Impact of TiO_2 NPs on plant (*M. truncatula*) growth; B: Impact of TiO_2 NPs on the
 204 number of plant leaves; C: Ti concentrations measured by ICP-OES in the aboveground biomass
 205 of plants; D: Principal component analysis indicating the impact of TiO_2 NPs on the elemental
 206 content in the leaves. Asterisks represent significant differences ($p < 0.05$). Average Ti
 207 concentrations in spiked soil = $341.7 \pm 12.2 \text{ mg kg}^{-1}$; average Ti concentrations in control soil =
 208 $278.0 \pm 13.4 \text{ mg kg}^{-1}$. Exposure: 60 days; $n = 10$.

209

210 The significant differences between non-exposed and exposed plants indicate an impact of TiO₂
211 NPs tending to inhibit plant growth and shoot biomass. Rafique *et al.*³⁷ showed that TiO₂ NP
212 concentrations of 20 to 60 mg kg⁻¹ (sandy loam) led to a dose-related increase in root and shoot
213 length and biomass in wheat plants exposed for 60 days. However, they demonstrated that
214 concentrations of 80 to 100 mg kg⁻¹ gradually inhibited root and shoot length and reduced plant
215 biomass. In our study, Ti concentrations were fivefold higher in the plants exposed (341.7 mg kg⁻¹)
216 for 60 days when compared with the non-exposed plants. Our results also conform to those
217 obtained in a recent study by Larue *et al.*, which showed that wheat plants exposed for 3 weeks
218 to sandy soils spiked with 500 mg kg⁻¹ of TiO₂ NPs took up significantly more Ti than the
219 control.¹¹ In addition, plants initially developing at the same rate began to show a consistent
220 change in growth rate by the beginning of the third week of exposure. We hypothesize that one
221 reason for the significant difference in plant development is the impact of TiO₂ NPs on plant
222 ionome. Among the deficient elements in plants grown in TiO₂ NP-spiked soils is potassium,
223 which is essential for plant growth, development, regulation, and crop yield.³⁸ Maathuis³⁹ posited
224 that at low potassium levels, sodium can replace potassium due to its structural similarity, which
225 is evidenced in our study, where the plants deficient in potassium are enriched, instead, in
226 sodium. Pi *et al.*⁴⁰ showed that when plants were exposed to stress (K deficient environment),
227 they replaced K⁺ with Na⁺, but did not recover to the same extent as the control group, which
228 could explain why the exposed plants were significantly smaller and less productive—with a
229 deficiency in potassium—than the non-exposed plants. However, contrary to our results, Servin
230 *et al.*⁴¹ demonstrated an increase in potassium in cucumber plants exposed to TiO₂ NPs. This
231 difference might be attributed to the plant models used. Also in line with our results, Rafique *et*
232 *al.*⁴² showed an increase in phosphorus availability in wheat plants exposed to TiO₂ NPs. In

233 addition, ICP analysis showed that spiked soil plants were deficient in other micronutrients, such
234 as boron, copper, iron, and molybdenum, which are necessary for the plant physiological
235 processes⁴³ and, therefore, could be explanative of the significant reduced development of the
236 exposed plants.

237

238 2.1.3. Impact of TiO₂ NPs on soil microbial communities

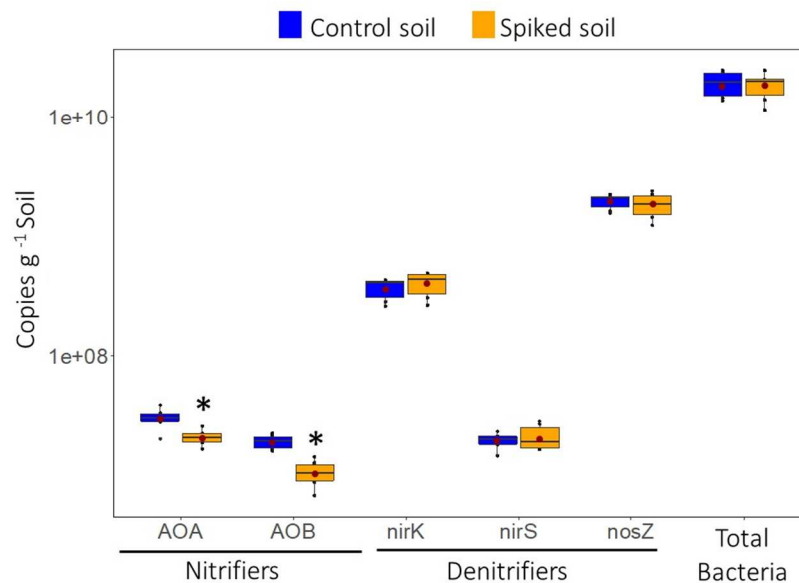
239 There was no difference between the abundance of the total bacteria (16S rRNA) in the control
240 and spiked soils ($p = 0.74$), with the average number of gene copies per gram of soil being $1.92 \times$
241 $10^{10} \pm 1.95 \times 10^9$ and $1.83 \times 10^{10} \pm 1.97 \times 10^9$, respectively (Figure 2).

242 Evaluation of *amoA* genes, both for AOA and AOB, indicated a significant decrease in
243 abundance in the spiked soil ($p = 0.007$ and $p = 0.001$, respectively). The average number of
244 gene copies/g soil was $2.97 \times 10^7 \pm 2.48 \times 10^6$ for non-exposed AOA and $2.07 \times 10^7 \pm 1.31 \times 10^6$
245 for exposed AOA, while those for non-exposed AOB and exposed AOB were $1.90 \times 10^7 \pm 1.04$
246 $\times 10^6$ and $1.05 \times 10^7 \pm 1.11 \times 10^6$, respectively (Figure 2).

247 In the case of the denitrifying genes *nirK*, *nirS*, and *nosZ*, no significant difference was found
248 between the control and spiked soils ($p = 0.49$, $p = 0.76$, and $p = 0.67$, respectively). The average
249 number of gene copies/g soil obtained for *nirK*, *nirS*, and *nosZ* were $3.81 \times 10^8 \pm 3.47 \times 10^7$, 2.00
250 $\times 10^7 \pm 1.80 \times 10^6$, and $1.91 \times 10^9 \pm 1.52 \times 10^8$, respectively (Figure 2).

251

252



253
 254 Figure 2: Impact on bacterial nitrifier and denitrifier genes. Nitrifier genes include the ammonia
 255 oxidizing archaea (AOA) and the ammonia oxidizing bacteria (AOB) ammonia monooxygenase
 256 A gene (*amoA*). The denitrifier genes include the bacterial nitrite reductase genes (*nirK* and *nirS*)
 257 and the bacterial nitrous oxide reductase gene (*nosZ*) for denitrification. Values on the y axis are
 258 expressed on the logarithm scale. Asterisks represent significant differences ($p < 0.05$). Average
 259 Ti concentrations in spiked soil = $341.7 \pm 12.2 \text{ mg kg}^{-1}$; average Ti concentrations in control soil
 260 = $278.0 \pm 13.4 \text{ mg kg}^{-1}$. Exposure: 60 days; $n = 6$.
 261

262 The literature concerning the impact of TiO₂ NPs on the soil microbial communities is scarce. Ge
 263 *et al.*^{44,45} and Simonin *et al.*⁴⁶ showed reductions in bacterial biomass and shifts in the
 264 community structure at high to very high TiO₂ NP concentrations (up to 20 g kg⁻¹). In our
 265 experiment, the total bacterial abundance was not affected probably because the TiO₂ NP
 266 concentration was lower. However, the relative abundance of some taxa decreased (soil nitrifiers
 267 vs. denitrifiers), suggesting potential consequences on bacterial community functionalities.
 268 Interestingly, Simonin *et al.* evidenced a lack of the classical dose–response relationship.⁴⁶ This
 269 phenomenon was explained by the concentration-dependent modifications of NP properties
 270 involved in their bioavailability and ecotoxicity.²⁵ Simonin *et al.*⁴⁶ showed a 40% reduction in
 271 AOA abundance at TiO₂ NP concentrations from 0.05 to 500 mg kg⁻¹. The magnitude of AOA
 272 decrease in our experiment was $35.9 \pm 4.5\%$, which is in agreement. In the case of AOB,

273 Simonin *et al.*⁴⁶ evidenced an unusual “U” shape for dose response, with intermediate
274 concentrations of TiO₂ NPs significantly decreasing the AOB abundance, and the lowest and
275 highest concentrations showing no significant decrease. Based on the model derived from their
276 experimental data ($y = 0.009 \times \ln ([\text{TiO}_2])^2 - 0.0215 \times \ln ([\text{TiO}_2]) - 0.26$), the estimation of AOB
277 decrease at 341 mg kg⁻¹ would be 6.5%. In our study, we measured a higher decrease: 44.9% ±
278 7.3%. While Simonin *et al.*⁴⁶ attributed the unusual dose-response relationships to the
279 modification of TiO₂ NP properties based on their concentrations (e.g., aggregation of TiO₂ NPs
280 at intermediate and higher concentrations), the discrepancy between results for AOB obtained in
281 their study and ours might be explained by the difference between soil types and thereby
282 between soil–NP interactions. Simonin *et al.*⁴⁶ used silty clay, whereas our study used silty sand,
283 highlighting the importance to consider soil type when studying NP fate in the environment.
284 Bissett *et al.*⁴⁷ discussed the relative insensitivity of denitrifiers to environmental toxicants, and
285 attributed the resistance of this functional guild to their high functional redundancy, niche
286 breadth, and adaptive ability compared with the soil nitrifiers, which have limited phylogenetic
287 diversity. In terms of relationship between plants and soil bacteria, we hypothesize that another
288 reason for the inhibition in plant development, and the delay of this impact until the third week,
289 could be an indirect effect of TiO₂ NPs on the soil nitrifier community. Indeed, Van Der
290 Heijden⁴⁸ discussed how soil bacteria indirectly affect plant productivity by altering nutrition and
291 resource availability.

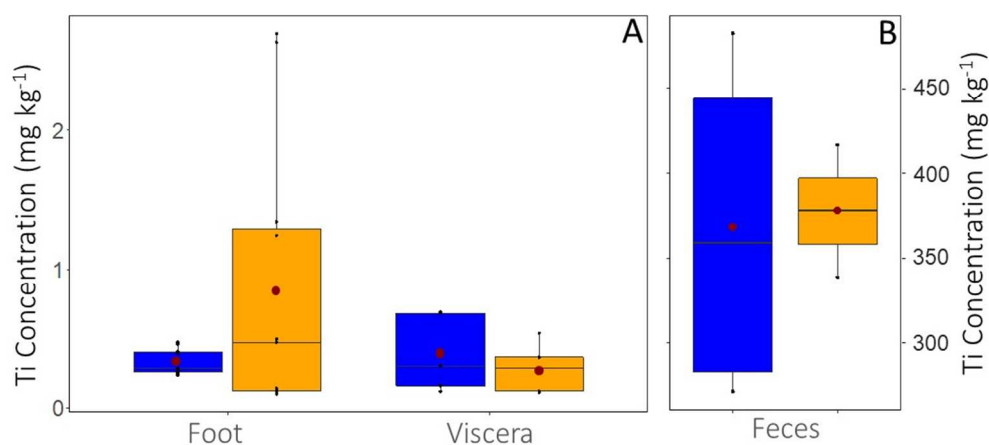
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293 2.1.4. Toxicity and uptake of Ti by *H. aspersa*

294 No difference in snail mortality was detected along the exposure period. The analysis of total soil
295 consumption by snails from day 1 to day 10 did not reveal any significant difference between

296 treatments ($p = 0.56$): the average daily soil consumed by snails amounting to 1.36 ± 0.17 g for
 297 spiked soil and to 1.23 ± 0.01 g for control soil (data not shown). Ti concentration in snail foot,
 298 viscera, and feces did not show significant difference ($p = 0.78, 0.49, 0.88$, respectively). Ti
 299 uptake in the non-exposed snail foot was 0.33 ± 0.03 mg kg⁻¹ and in the exposed snail foot was
 300 0.86 ± 0.30 mg kg⁻¹ (Figure 3A), while for the viscera, the average Ti concentrations after
 301 depuration were 0.39 ± 0.12 mg kg⁻¹ and 0.28 ± 0.08 mg kg⁻¹ for non-exposed and exposed
 302 snails, respectively (Figure 3A). Average Ti concentrations in feces were 367.78 ± 52.41 mg kg⁻¹
 303 for non-exposed snails and 377.51 ± 38.88 mg kg⁻¹ for exposed snails (Figure 3B).

304



305 Figure 3: A: Ti uptake in snail (*H. aspersa*) foot and viscera; B: Ti concentration in snail feces,
 306 measured by ICP-OES. Average Ti concentrations in spiked soil = 341.7 ± 12.2 mg kg⁻¹; average
 307 Ti concentrations in control soil = 278.0 ± 13.4 mg kg⁻¹. Snails were exposed to 5 g each of
 308 control or spiked soils every day. Exposure: 10 days; $n = 6$.

310

311 The total soil consumed by snails in the two treatments was not significantly different, which
 312 highlights that there was no behavior of avoidance of the contaminated soil. There was also no
 313 significant difference in sequestration of Ti from soil into snail foot and viscera, or in the
 314 excretion in feces. Concentrations in the snail feces show that Ti was quickly eliminated from the
 315 snail body via feces. The higher concentrations of Ti in the feces of both the control and spiked

316 conditions compared to the soil concentrations is presumably due to the digestion of soil organic
317 matter, and thus to a concentration of its mineral fraction. While results on the effects on land
318 snails are in lack, Yeo and Nam¹⁹ performed a paddy microcosm study, in which TiO₂ NPs were
319 found to transfer from prey to consumer (biofilm/water dropwort to nematodes/snail) within a
320 period of 17 days, highlighting the potential for bioaccumulation in the food chain. In 2006,
321 Scheifler *et al.*⁴⁹ evaluated the transfer of several heavy metals in a soil–plant–invertebrate
322 microcosm, using lettuce as primary producer and the snail *H. aspersa* as primary consumer.
323 They demonstrated that while copper and nickel were not accumulated in the snail body, there
324 was a gradual increase in cadmium and zinc, and a rapid accumulation of lead in snails, but more
325 than 80% of this accumulation was contributed from soil exposure rather than plant exposure.
326 Similarly, other studies have demonstrated significant increases in concentrations of metals such
327 as chromium and platinum from contaminated food in *H. aspersa* after 56 days⁵⁰. Although our
328 results did not show any significant uptake in snail, there was a trend for higher Ti
329 concentrations in snail foot under NP exposure, *ca.* two-fold higher on average than in controls.
330 The absence of a significant effect may be due to the high variability in the concentrations
331 measured, reducing statistical power. Including more replicates and a longer exposure period
332 may be needed to reach a conclusion.

333

334 2.2. Aquatic ecosystem

335 2.2.1. Ti in amphibian exposure medium

336 Titanium in the amphibian exposure media showed a higher concentration of $12.3 \pm 0.15 \text{ mg L}^{-1}$
337 in the raw spiked soil leachates compared to $3.6 \pm 0.18 \text{ mg L}^{-1}$ in the raw control soil leachates (*p*

338 = 0.01). Whereas the ratio of Ti found in control soil versus spiked soil was 1:1.3, the ratio of Ti
339 found in control soil leachates versus spiked soil leachates was 1:3.4.

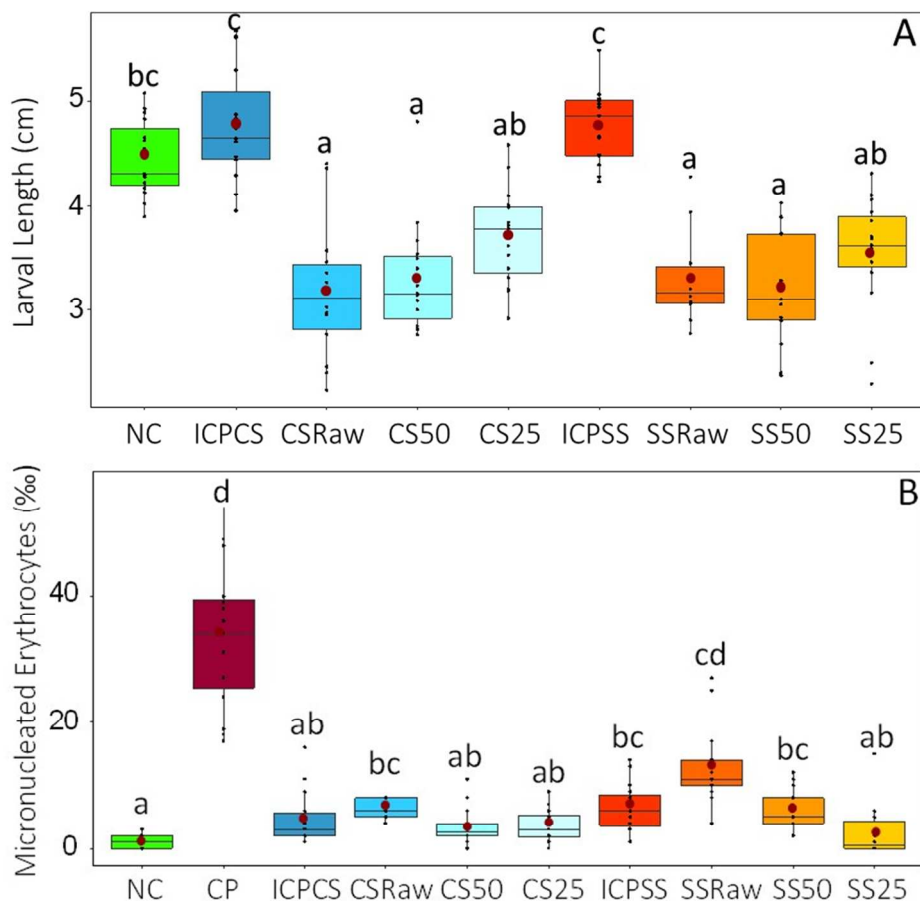
340 In the present study, a real environmental exposure model in the aquatic system was designed,
341 taking into account an approximation of expected percentage of TiO₂ NP concentrations coming
342 from the leaching of an agricultural soil into surface waters. Leachate Ti concentrations were
343 much higher than the predicted concentrations of 2.17 μg L⁻¹ TiO₂ NPs in surface waters by Sun
344 *et al.* (2016), which is attributed to the high concentration of soil spiking in our study. The
345 increased ratios of Ti in the spiked soil leachates compared with the control soil leachates mean
346 that more Ti from the spiked soil moved into the water compartment during the preparation of
347 soil leachates than from the control soil. Gogos *et al.*⁵¹ showed a significantly lower transfer of
348 Ti from contaminated soil to soil leachate (0.001% vs. 26% in our study) but with a different
349 leaching procedure (filtration of the NP suspension through the soil layer vs. run-off simulation
350 over a soil including soil particles with no filtering step).

351

352 2.2.2. Impact of TiO₂ NPs on *Xenopus laevis* larvae

353 There was no significant mortality (<20%) among amphibian larvae exposed to TiO₂ NPs. A
354 comparison of the larval lengths after 12 days of exposure showed a significant difference among
355 groups ($p < 0.0001$) (Figure 4A). There was no significant decrease in the length of the larvae
356 exposed to the ICP equivalents of both the spiked and control soil leachates compared to the
357 negative control larvae (4.77 ± 0.09 cm and 4.77 ± 0.14 cm, respectively, vs. 4.46 ± 0.09 cm).
358 Larvae exposed to raw spiked and control soil leachates (3.31 ± 0.14 cm and 3.17 ± 0.17 cm,
359 respectively) and 50% diluted spiked and control leachates (3.21 ± 0.14 cm and 3.30 ± 0.13 cm,

360 respectively) showed significant lower size compared to the negative control larvae and to the
361 both ICP equivalents. Larvae exposed to 25% diluted spiked and control soil leachates also
362 showed a significantly reduced size (3.54 ± 0.14 cm and 3.70 ± 0.11 cm, respectively) compared
363 to both the ICP equivalents, but no significant lower length compared to the negative control
364 larvae. In addition, the larval size tended to increase with the leachate dilution. There was a
365 significant genotoxicity ($p < 0.0001$ among groups) in larvae exposed to the ICP equivalent of
366 the spiked soil leachate (6.60 ± 0.97 micronuclei), to the spiked and control soil raw leachates
367 (13.15 ± 1.81 and 6.08 ± 0.49 micronuclei, respectively), and to the 50% diluted spiked soil
368 leachate (6.07 ± 0.90 micronuclei), compared to the negative control larvae (1.07 ± 0.30
369 micronucleus) (Figure 4B). There was no significant genotoxicity in larvae exposed to the ICP
370 equivalent of the control soil leachate (4.73 ± 1.09 micronuclei), to the 50% diluted control soil
371 leachate (3.42 ± 0.82 micronuclei), and to the 25% diluted spiked and control soil leachates (2.57
372 ± 1.11 and 3.50 ± 0.81 micronuclei, respectively) compared to the negative control larvae.



373
 374 Figure 4: A: Impact of Ti on amphibian (*X. laevis*) larval length; B: Percentage of
 375 micronucleated erythrocytes (ME%). Average Ti concentration in the control soil leachates was
 376 3.6 mg L^{-1} and that in the spiked soil leachates was 12.6 mg L^{-1} . Exposure: 12 days; $n = 15$. NC =
 377 negative control; PC = positive control; ICP CS = ICP equivalent of control soil leachates (3.6
 378 mg L^{-1} TiO_2 NPs in Volvic®); CS Raw = raw control soil leachates; CS 50% = 50% diluted
 379 control soil leachates in Volvic®; CS 25% = 25% diluted control soil leachates in Volvic®; ICP
 380 SS = ICP equivalent of spiked soil leachates (12.6 mg L^{-1} TiO_2 NPs in Volvic®); SS Raw = raw
 381 spiked soil leachates; SS50% = 50% diluted spiked soil leachates in Volvic®; SS 25% = 25%
 382 diluted spiked soil leachates in Volvic® water. Alphabets represent statistically different groups
 383 ($p < 0.05$).

384 Our results demonstrated the inhibition potential of soil leachates on larval growth, and a
 385 genotoxicity of both soil leachates and TiO_2 NPs. Mouchet *et al.*²⁹ already demonstrated a
 386 toxicity of leachates from contaminated soil to amphibian larvae. Although Chenon *et al.*⁵²
 387 showed that sludge-amended soil leachates, including non-lethal dilutions, lead to a decrease in
 388 size of amphibian larvae, in our study, the impact of raw control soil leachates was an

389 unexpected finding. Therefore, we performed an ICP-OES analysis separately on the compost for
390 elemental concentrations. The results showed an average lead concentration of 9.8 mg kg^{-1} ,
391 which might have resulted in a cocktail effect with TiO_2 NPs, as Mouchet *et al.*⁵³ found lead
392 concentrations from 1 mg L^{-1} to be genotoxic to amphibian larvae. In addition, according to
393 Bekaert *et al.*,⁵⁴ pollutants adsorbed onto soil particles may be biologically active when
394 compared with those on filtrates, and as a result, in our study, the presence of soil particles may
395 have contributed to the impact on amphibian growth. The genotoxic potential of TiO_2 NPs was
396 evidenced by the increase in the number of micronucleated erythrocytes in larvae exposed to the
397 ICP equivalent of/and the raw spiked soil leachates. Bacchetta *et al.*⁵⁵ also demonstrated the
398 toxicity of TiO_2 NPs in *X. laevis* when exposed for 96 h to concentrations ranging from 10 to 500
399 mg L^{-1} , resulting in deformities in embryo development. Previous studies have already shown the
400 negative effects of TiO_2 NPs on aquatic organisms such as *X. laevis*⁵⁶ and *Danio rerio*,⁵⁷ causing
401 deformities and death, while Federici *et al.*⁵⁸ showed that TiO_2 NPs had toxic effects in rainbow
402 trout at concentrations $<1.0 \text{ mg L}^{-1}$, which could indicate a difference in sensitivities among
403 species. The ICP equivalents of both the raw control soil and raw spiked soil leachates had half
404 or less than half the micronucleated erythrocytes compared with both the raw leachates,
405 respectively, which, again, could be due to a cocktail effect resulting from the presence of lead in
406 the compost.

407 Our study highlights the potential risks of TiO_2 NPs in the environment, showing an upward
408 transfer of Ti in the terrestrial ecosystem, from soil to plants, and a downward transfer of Ti from
409 soil to the amphibian exposure medium. The inhibition of nitrifiers due to the presence of TiO_2
410 NPs can have far-reaching consequences, especially for soil fertility, global ecosystem
411 functions/services, and crop production. The indirect effect of nitrifiers on crop production, and

412 the uptake of Ti by alfalfa plants, lead to the question of food security. Although we did not find
413 significant uptake of Ti by snails, the concentration-dependent sequestration of Ti in snail foot
414 deserves some attention with the possibility for transfer up the food chain and bioaccumulation
415 over the years. Finally, runoff from contaminated soils ends up in freshwater ecosystems and can
416 be genotoxic to amphibian larvae. By focusing on several experimental models rather than a
417 single-species study, we provide data concerning the distribution of TiO₂ NPs between the
418 terrestrial and aquatic ecosystems, the levels of the terrestrial ecosystem that are most affected—
419 soil bacteria and primary producers—and the risk of toxicity in aquatic ecosystems by leaching
420 of Ti from soil. Three-fourths of our experimental models have been affected by TiO₂ NPs,
421 which is sufficient to acknowledge that precautionary standards for TiO₂ NPs are required. These
422 ecotoxicological effects can be used in future studies to identify safe threshold values of TiO₂
423 NPs in the environment and suitable waste disposal methods to ensure that NPs do not reach
424 such high concentrations as identified to be toxic in our study.

425

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429

430 **Supporting Information Available**

431 This information is available free of charge via the Internet at <http://pubs.acs.org>.

432

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