



**HAL**  
open science

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

Vinita Vijayaraj, Clarisse Liné, Stéphanie Cadarsi, Clément Salvagnac, David Baqué, Arnaud Elger, Maialen Barret, Florence Mouchet, Camille Larue

► **To cite this version:**

Vinita Vijayaraj, Clarisse Liné, Stéphanie Cadarsi, Clément Salvagnac, David Baqué, et al.. Transfer and Ecotoxicity of Titanium Dioxide Nanoparticles in Terrestrial and Aquatic Ecosystems: A Microcosm Study. *Environmental Science and Technology*, 2018, 52 (21), pp.12757-12764. 10.1021/acs.est.8b02970 . hal-02325006

**HAL Id: hal-02325006**

**<https://hal.science/hal-02325006>**

Submitted on 10 Nov 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

This document is confidential and is proprietary to the American Chemical Society and its authors. Do not copy or disclose without written permission. If you have received this item in error, notify the sender and delete all copies.

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

Journal:	<i>Environmental Science &amp; Technology</i>
Manuscript ID	es-2018-02970a.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Vijayaraj, Vinita; CNRS, ECOLAB Liné, Clarisse; CNRS, ECOLAB Cadarsi, Stéphanie; CNRS, ECOLAB Salvagnac, Clément; CNRS, ECOLAB Baqué, David; CNRS, ECOLAB Elger, Arnaud; EcoLab Barret, Maialen; CNRS, ECOLAB Mouchet, Florence; Université de Toulouse, EcoLab Larue, Camille; CNRS, ECOLAB

SCHOLARONE™  
Manuscripts

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

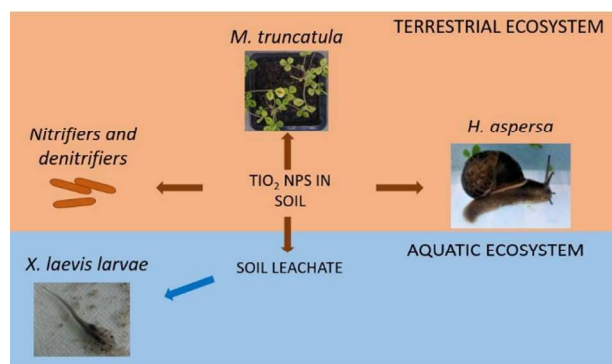
5

6 <sup>†</sup>**EcoLab, Université de Toulouse, CNRS, Toulouse, France**

7

8

9

10 **Graphical abstract**

11

12

13 **ABSTRACT:** With the advancement in nanotechnology, particularly the use of TiO<sub>2</sub>  
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 TiO<sub>2</sub> 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 TiO<sub>2</sub> 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.<sup>1</sup> Compared  
28 with their micro/macro particle counterparts, NPs have a larger surface area over volume ratio,<sup>2</sup>  
29 and number of surface atoms, which results in increased surface energy and reactivity.<sup>3</sup> 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,<sup>4</sup> they  
33 could pose different potential hazards.<sup>5,6</sup> Despite the recommendation by the National Organic  
34 Standards Board in 2010, products containing NPs are still being manufactured worldwide.<sup>4</sup> 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 (TiO<sub>2</sub>) NPs.<sup>7</sup> TiO<sub>2</sub> NPs are  
37 commonly known for their photocatalytic applications and used as semiconductors,<sup>8</sup> additives in  
38 food,<sup>9</sup> and in cosmetic products.<sup>10</sup> Ti is also a widespread element in the environment, with  
39 concentrations in soils ranging for instance from 43 mg kg<sup>-1</sup> in sand to 1671 mg kg<sup>-1</sup> in clayey  
40 soil.<sup>11</sup> 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.<sup>12</sup> 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%)<sup>13</sup> that remains in sewage sludge is dispersed  
46 into agricultural fields as fertilizer.<sup>14</sup> Runoff from these polluted soils can in turn reach water  
47 bodies. A recent study by Sun *et al.*<sup>15</sup> predicted the concentrations of TiO<sub>2</sub> NPs in surface waters  
48 to be ~ 2.17 µg L<sup>-1</sup>, in sludge 1.60 g kg<sup>-1</sup>, and in sludge-treated soils 61.10 mg kg<sup>-1</sup>. 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,<sup>16</sup> 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.<sup>17,18</sup> Yeo and Nam<sup>19</sup> compared the bioaccumulation of TiO<sub>2</sub> 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.*<sup>20</sup>  
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.*<sup>21</sup> showed that this ecosystem function was not impacted by CeO<sub>2</sub> 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 TiO<sub>2</sub> 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 TiO<sub>2</sub> 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 TiO<sub>2</sub>

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. TiO<sub>2</sub> NP characterization and suspension preparation

76 TiO<sub>2</sub> 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  $25.0 \pm 5.7$  nm; are polyhedral, uncoated, with a specific surface  
79 area of  $46 \pm 1$  m<sup>2</sup> g<sup>-1</sup> and zeta potential in ultrapure water of  $-10.5 \pm 2.6$  mV (File S1).<sup>22</sup> The  
80 TiO<sub>2</sub> NP suspension was freshly prepared before soil contamination (10000 mg L<sup>-1</sup>); 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<sup>-1</sup> of TiO<sub>2</sub> NPs were added to mimic  
89 several years of sewage sludge spreading on agricultural soils.<sup>15</sup> 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 TiO<sub>2</sub> NPs

96 *Medicago truncatula* (ecotype A17) is a model for legume plants,<sup>23</sup> chosen for their implication  
97 in the nitrogen cycle.<sup>24</sup> 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 TiO<sub>2</sub> 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.<sup>25</sup> For more details about the experimental  
119 procedure, see File S3.

120

#### 121 1.2.4. *Helix aspersa* exposure to TiO<sub>2</sub> NPs

122 Snails were chosen for this experiment as sensitive bioindicators of pollution in the  
123 environment.<sup>26</sup> 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.<sup>26</sup>

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.<sup>29</sup> 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 <sup>-1</sup> cyclophosphamide in Volvic® water
ICP equiv of CS	3.6 mg L <sup>-1</sup> TiO <sub>2</sub> 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 <sup>-1</sup> TiO <sub>2</sub> 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 TiO<sub>2</sub> NPs on *Xenopus laevis*

146 *X. laevis*, a widely used model in ecotoxicology,<sup>27,28</sup> 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 & Faber<sup>30</sup>) were exposed in semi-static conditions based on  
149 modified ISO 21427-1.<sup>31</sup> 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 test<sup>31</sup>: 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 HNO<sub>3</sub>, NH<sub>4</sub>F, and HClO<sub>4</sub> as well  
159 as H<sub>2</sub>O<sub>2</sub>, with a temperature of 100°C and several evaporation steps.<sup>11</sup> Digested samples were  
160 diluted in 5% HNO<sub>3</sub>. 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 TiO<sub>2</sub> NPs has been calculated to be  $88.2 \pm 2.1\%$ .<sup>11</sup>

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 TiO<sub>2</sub> NP impact on plant  
170 nutrition. All statistical analyses were performed using the R statistical software (version  
171 3.1.3),<sup>32</sup> with `car`,<sup>33</sup> `multcompView`,<sup>34</sup> `pgirmess`,<sup>35</sup> and `lsmeans`<sup>36</sup> 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 TiO<sub>2</sub> 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).

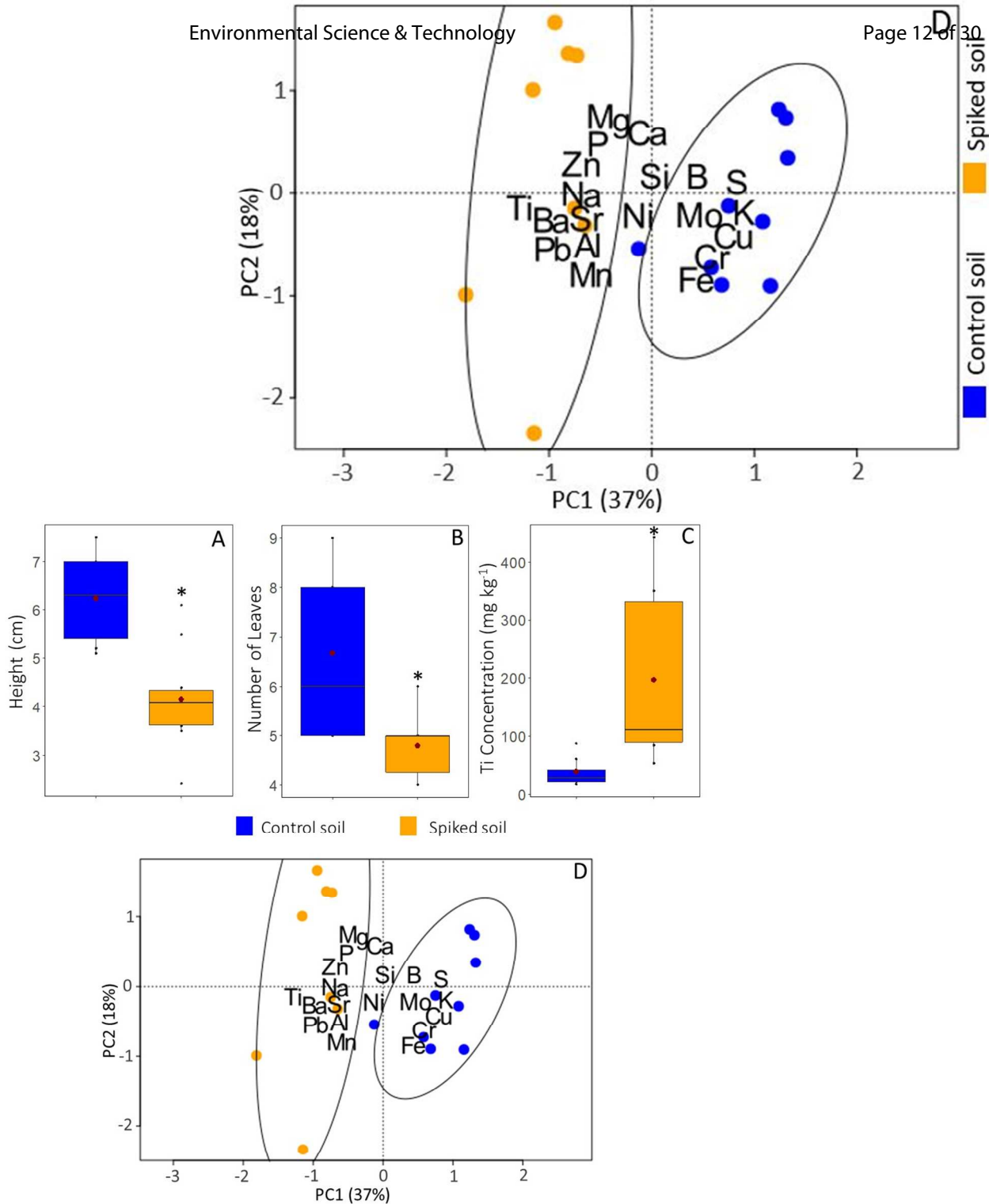
181 Although the soils were spiked with  $500 \text{ mg kg}^{-1}$  TiO<sub>2</sub> NPs ( $300 \text{ mg Ti kg}^{-1}$  equivalent), the  
182 average increased concentration of Ti in spiked soils after leaching was below  $300 \text{ mg kg}^{-1}$  Ti.  
183 Considering that the soil used in this study 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.<sup>11</sup>

186

#### 187 2.1.2. TiO<sub>2</sub> 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  $196.6 \pm 53.1$   
198  $\text{mg kg}^{-1}$  and  $37.6 \pm 7.9 \text{ mg kg}^{-1}$  (Figure 1C). Exposure to  $\text{TiO}_2$  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  $\text{TiO}_2$  NPs on plant (*M. truncatula*) growth; B: Impact of  $\text{TiO}_2$  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  $\text{TiO}_2$  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 TiO<sub>2</sub>  
211 NPs tending to inhibit plant growth and shoot biomass. Rafique *et al.*<sup>37</sup> showed that TiO<sub>2</sub> NP  
212 concentrations of 20 to 60 mg kg<sup>-1</sup> (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<sup>-1</sup> 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<sup>-1</sup>)  
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<sup>-1</sup> of TiO<sub>2</sub> NPs took up significantly more Ti than the  
219 control.<sup>11</sup> 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 TiO<sub>2</sub> NPs on plant  
222 ionome. Among the deficient elements in plants grown in TiO<sub>2</sub> NP-spiked soils is potassium,  
223 which is essential for plant growth, development, regulation, and crop yield.<sup>38</sup> Maathuis<sup>39</sup> 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.*<sup>40</sup> showed that when plants were exposed to stress (K deficient environment),  
227 they replaced K<sup>+</sup> with Na<sup>+</sup>, 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.*<sup>41</sup> demonstrated an increase in potassium in cucumber plants exposed to TiO<sub>2</sub> NPs. This  
231 difference might be attributed to the plant models used. Also in line with our results, Rafique *et*  
232 *al.*<sup>42</sup> showed an increase in phosphorus availability in wheat plants exposed to TiO<sub>2</sub> 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 processes<sup>43</sup> and, therefore, could be explanative of the significant reduced development of the  
236 exposed plants.

237

### 238 2.1.3. Impact of TiO<sub>2</sub> 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).

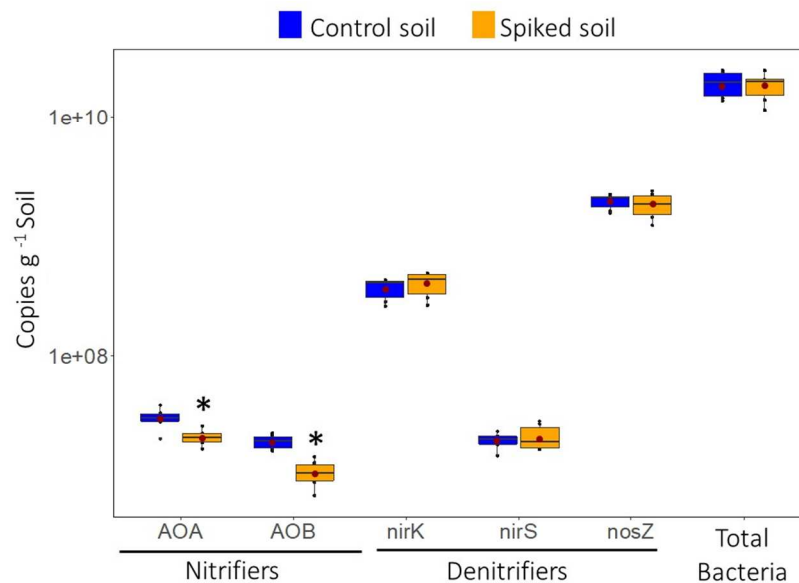
242 Evaluation of *amoA* genes, both for AOA and AOB, indicated a significant decrease in  
243 abundance in the spiked soil ( $p = 0.007$  and  $p = 0.001$ , respectively). The average number of  
244 gene copies/g soil was  $2.97 \times 10^7 \pm 2.48 \times 10^6$  for non-exposed AOA and  $2.07 \times 10^7 \pm 1.31 \times 10^6$   
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 TiO<sub>2</sub> NPs on the soil microbial communities is scarce. Ge  
 263 *et al.*<sup>44,45</sup> and Simonin *et al.*<sup>46</sup> showed reductions in bacterial biomass and shifts in the  
 264 community structure at high to very high TiO<sub>2</sub> NP concentrations (up to 20 g kg<sup>-1</sup>). In our  
 265 experiment, the total bacterial abundance was not affected probably because the TiO<sub>2</sub> 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.<sup>46</sup> This  
 269 phenomenon was explained by the concentration-dependent modifications of NP properties  
 270 involved in their bioavailability and ecotoxicity.<sup>25</sup> Simonin *et al.*<sup>46</sup> showed a 40% reduction in  
 271 AOA abundance at TiO<sub>2</sub> NP concentrations from 0.05 to 500 mg kg<sup>-1</sup>. 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.*<sup>46</sup> evidenced an unusual “U” shape for dose response, with intermediate  
274 concentrations of TiO<sub>2</sub> 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<sup>-1</sup> would be 6.5%. In our study, we measured a higher decrease: 44.9% ±  
278 7.3%. While Simonin *et al.*<sup>46</sup> attributed the unusual dose-response relationships to the  
279 modification of TiO<sub>2</sub> NP properties based on their concentrations (e.g., aggregation of TiO<sub>2</sub> 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.*<sup>46</sup> 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.*<sup>47</sup> 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 TiO<sub>2</sub> NPs on the soil nitrifier community. Indeed, Van Der  
290 Heijden<sup>48</sup> discussed how soil bacteria indirectly affect plant productivity by altering nutrition and  
291 resource availability.

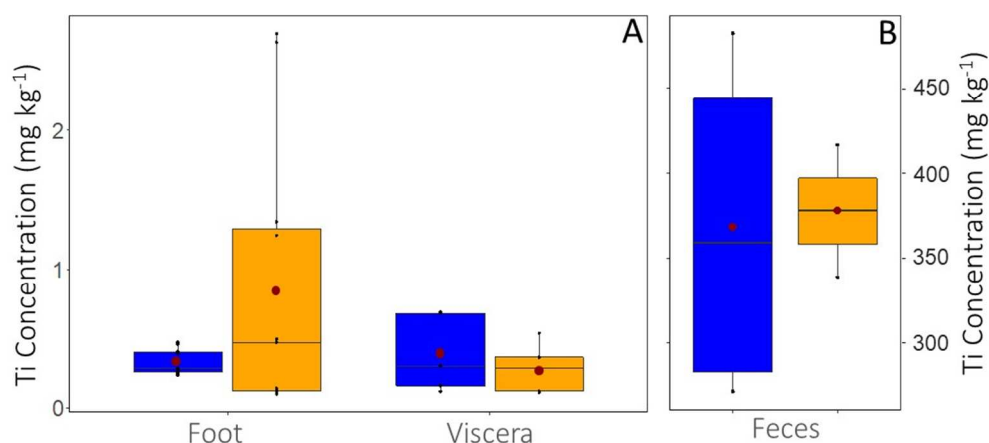
292

#### 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  $1.36 \pm 0.17$  g for  
 297 spiked soil and to  $1.23 \pm 0.01$  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  $0.33 \pm 0.03$  mg kg<sup>-1</sup> and in the exposed snail foot was  
 300  $0.86 \pm 0.30$  mg kg<sup>-1</sup> (Figure 3A), while for the viscera, the average Ti concentrations after  
 301 depuration were  $0.39 \pm 0.12$  mg kg<sup>-1</sup> and  $0.28 \pm 0.08$  mg kg<sup>-1</sup> for non-exposed and exposed  
 302 snails, respectively (Figure 3A). Average Ti concentrations in feces were  $367.78 \pm 52.41$  mg kg<sup>-1</sup>  
 303 for non-exposed snails and  $377.51 \pm 38.88$  mg kg<sup>-1</sup> 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 =  $341.7 \pm 12.2$  mg kg<sup>-1</sup>; average  
 307 Ti concentrations in control soil =  $278.0 \pm 13.4$  mg kg<sup>-1</sup>. 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 Nam<sup>19</sup> performed a paddy microcosm study, in which TiO<sub>2</sub> 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.*<sup>49</sup> 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 days<sup>50</sup>. 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.

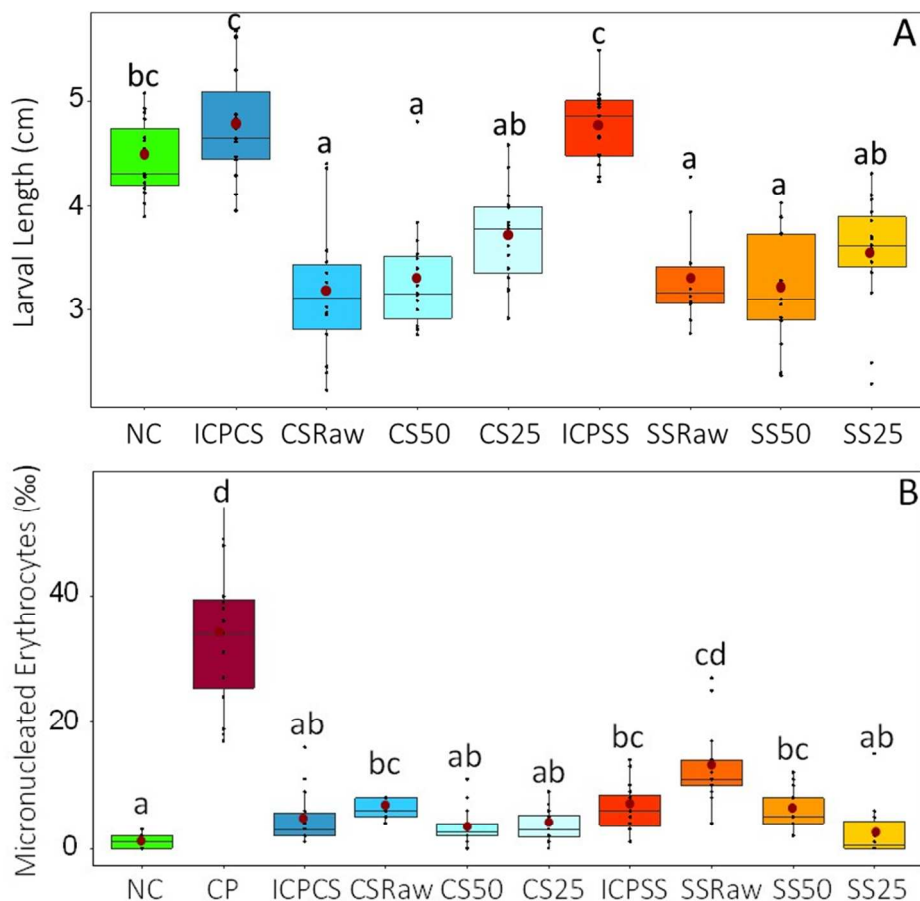
340 In the present study, a real environmental exposure model in the aquatic system was designed,  
341 taking into account an approximation of expected percentage of TiO<sub>2</sub> NP concentrations coming  
342 from the leaching of an agricultural soil into surface waters. Leachate Ti concentrations were  
343 much higher than the predicted concentrations of 2.17 μg L<sup>-1</sup> TiO<sub>2</sub> 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.*<sup>51</sup> 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 TiO<sub>2</sub> NPs on *Xenopus laevis* larvae

353 There was no significant mortality (<20%) among amphibian larvae exposed to TiO<sub>2</sub> 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 ( $4.77 \pm 0.09$  cm and  $4.77 \pm 0.14$  cm, respectively, vs.  $4.46 \pm 0.09$  cm).  
358 Larvae exposed to raw spiked and control soil leachates ( $3.31 \pm 0.14$  cm and  $3.17 \pm 0.17$  cm,  
359 respectively) and 50% diluted spiked and control leachates ( $3.21 \pm 0.14$  cm and  $3.30 \pm 0.13$  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 ( $3.54 \pm 0.14$  cm and  $3.70 \pm 0.11$  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 ( $6.60 \pm 0.97$  micronuclei), to the spiked and control soil raw leachates  
367 ( $13.15 \pm 1.81$  and  $6.08 \pm 0.49$  micronuclei, respectively), and to the 50% diluted spiked soil  
368 leachate ( $6.07 \pm 0.90$  micronuclei), compared to the negative control larvae ( $1.07 \pm 0.30$   
369 micronucleus) (Figure 4B). There was no significant genotoxicity in larvae exposed to the ICP  
370 equivalent of the control soil leachate ( $4.73 \pm 1.09$  micronuclei), to the 50% diluted control soil  
371 leachate ( $3.42 \pm 0.82$  micronuclei), and to the 25% diluted spiked and control soil leachates ( $2.57$   
372  $\pm 1.11$  and  $3.50 \pm 0.81$  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  $3.6 \text{ mg L}^{-1}$  and that in the spiked soil leachates was  $12.6 \text{ mg L}^{-1}$ . Exposure: 12 days;  $n = 15$ . NC =  
 377 negative control; PC = positive control; ICP CS = ICP equivalent of control soil leachates ( $3.6$   
 378  $\text{mg L}^{-1}$   $\text{TiO}_2$  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 ( $12.6 \text{ mg L}^{-1}$   $\text{TiO}_2$  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  $\text{TiO}_2$  NPs. Mouchet *et al.*<sup>29</sup> already demonstrated a  
 386 toxicity of leachates from contaminated soil to amphibian larvae. Although Chenon *et al.*<sup>52</sup>  
 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  $9.8 \text{ mg kg}^{-1}$ ,  
391 which might have resulted in a cocktail effect with  $\text{TiO}_2$  NPs, as Mouchet *et al.*<sup>53</sup> found lead  
392 concentrations from  $1 \text{ mg L}^{-1}$  to be genotoxic to amphibian larvae. In addition, according to  
393 Bekaert *et al.*,<sup>54</sup> 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  $\text{TiO}_2$  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.*<sup>55</sup> also demonstrated the  
398 toxicity of  $\text{TiO}_2$  NPs in *X. laevis* when exposed for 96 h to concentrations ranging from 10 to 500  
399  $\text{mg L}^{-1}$ , resulting in deformities in embryo development. Previous studies have already shown the  
400 negative effects of  $\text{TiO}_2$  NPs on aquatic organisms such as *X. laevis*<sup>56</sup> and *Danio rerio*,<sup>57</sup> causing  
401 deformities and death, while Federici *et al.*<sup>58</sup> showed that  $\text{TiO}_2$  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  $\text{TiO}_2$  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  $\text{TiO}_2$   
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 TiO<sub>2</sub> 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 TiO<sub>2</sub> NPs,  
421 which is sufficient to acknowledge that precautionary standards for TiO<sub>2</sub> NPs are required. These  
422 ecotoxicological effects can be used in future studies to identify safe threshold values of TiO<sub>2</sub>  
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

## 426 **ACKNOWLEDGMENT**

427 The authors thank Catherine and Marc Mage, from “L’Escargot de Barjac,” for having provided  
428 live snails.

429

## 430 **Supporting Information Available**

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

432

## 433 REFERENCES

- 434 (1) European Commission.  
435 [http://ec.europa.eu/environment/chemicals/nanotech/faq/definition\\_en.htm](http://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm).
- 436 (2) Guisbiers, G. Size-dependent materials properties toward a universal equation. *Nanoscale*  
437 *Res Lett*, **2010**, 5 (7), 1132–1136. doi: 10.1007/s11671-010-9614-1
- 438 (3) Banfield, J. F.; Navrotsky, A. *Nanoparticles and the Environment*; Mineralogical Society of  
439 America: Washington, DC, 2001.
- 440 (4) Kessler, R. Engineered nanoparticles in consumer products: understanding a new  
441 ingredient. *Environ Health Perspect.* **2011**, 119(3), A120–A125.
- 442 (5) SCENHIR. Opinion on: the appropriateness of the risk assessment methodology in  
443 accordance with the technical guidance documents for the new and existing substances for  
444 assessing the risk of nanomaterials. Brussels.
- 445 (6) Mitrano, D. M.; Motellier, S.; Clavaguera, S.; Nowack, B. *Environ Int.* **2015**, 77, 132–147.
- 446 (7) Mueller, N. C.; Nowack, B. Exposure modeling of engineered nanoparticles in the  
447 environment. *Environ Sci Technol.* **2008**, 42, 4447–4453.
- 448 (8) Gupta, S. M.; Tripathi, M. A review of TiO<sub>2</sub> nanoparticles. *Chin. Sci. Bull.* **2011**, 56 (16),  
449 1639.
- 450 (9) Ropers, M.-H.; Terrisse, H.; Mercier-Bonin, M.; Humbert, B. Titanium Dioxide as Food  
451 Additive. In M. Janus (Ed.), *Application of Titanium Dioxide*. InTech, 2017. Retrieved from  
452 [http://www.intechopen.com/books/application-of-titanium-dioxide/titanium-dioxide-as-food-](http://www.intechopen.com/books/application-of-titanium-dioxide/titanium-dioxide-as-food-additive)  
453 additive
- 454 (10) Lu, P.-J.; Huang, S.-C.; Chen, Y.-P.; Chiueh, L.-C.; Shih, D. Y.-C. Analysis of titanium  
455 dioxide and zinc oxide nanoparticles in cosmetics. *Water Air Soil Pollut.* **2015**, 23 (3), 587–594.

- 456 (11) Larue, C.; Baratange, C.; Vantelon, D.; Khodja, H.; Surblé, S.; Elger, A.; Carrière, M.  
457 Influence of soil type on TiO<sub>2</sub> nanoparticle fate in an agro-ecosystem. *Sci. Total Environ.* **2018**,  
458 *630*, 609–617.
- 459 (12) Kiser, M. A.; Westerhoff, P.; Benn, T.; Wang, Y.; Pérez-Rivera J.; Hristovski, K. Titanium  
460 nanomaterial removal and release from wastewater treatment plants. *Environ Sci Technol.* **2009**,  
461 *439*(17), 6757–6763.
- 462 (13) Johnson, A. C.; Bowes, M. J.; Crossley, A.; Jarvie, H. P.; Jurkschat, K.; Jürgens, M. D.;  
463 ... Xu, N. An assessment of the fate, behaviour and environmental risk associated with sunscreen  
464 TiO<sub>2</sub> nanoparticles in UK field scenarios. *Sci. Total Environ.* **2011**, *409* (13), 2503–2510.
- 465 (14) Weir, A.; Westerhoff, P.; Fabricius, L.; von Goetz, N. Titanium dioxide nanoparticles in  
466 food and personal care products. *Environ Sci Technol* **2012**, *46* (4), 2242–2250.
- 467 (15) Sun, T. Y.; Bornhöft, N. A.; Hungerbühler, K.; Nowack, B. Dynamic probabilistic  
468 modeling of environmental emissions of engineered nanomaterials. *Environ Sci Technol* **2016**,  
469 *50*, 4701–4711.
- 470 (16) Maurer-Jones, M. A.; Gunsolus, I. L.; Murphy, C. J.; Haynes, C. L. Toxicity of  
471 engineered nanoparticles in the environment. *Anal. Chem.* **2013**, *85*, 3036–3049.
- 472 (17) Nam, D.-H.; Lee, B.; Eom, I.; Kim, P.; Yeo, M.-K. Uptake and bioaccumulation of  
473 titanium- and silver-nanoparticles in aquatic ecosystems. *Mol Cell Toxicol* **2014**, *10* (1), 9–17.
- 474 (18) Gardea-Torresdey, J. L.; Rico, C. M.; White, J. C. Trophic Transfer, Transformation, and  
475 Impact of Engineered Nanomaterials in Terrestrial Environments. *Environ Sci Technol.* **2014**, *48*  
476 (5), 2526–2540.
- 477 (19) Yeo, M.-K.; Nam, D.-H. Influence of different types of nanomaterials on their  
478 bioaccumulation in a paddy microcosm: a comparison of TiO<sub>2</sub> nanoparticles and nanotubes.

- 479 *Environ. Pollut.* **2013**, *178*, 166–172.
- 480 (20) Servin, A. D.; Pagano, L.; Castillo-Michel, H.; De la Torre-Roche, R.; Hawthorne, J.;  
481 Hernandez-Viezcas, J. A.; ... White, J. C. Weathering in soil increases nanoparticle CuO  
482 bioaccumulation within a terrestrial food chain. *Nanotoxicology* **2017**, *11* (1), 98–111.
- 483 (21) Bour, A.; Mouchet, F.; Cadarsi, S.; Silvestre, J.; Chauvet, E.; Bonzom, J.-M.; ... Pinelli,  
484 E. Impact of CeO<sub>2</sub> nanoparticles on the functions of freshwater ecosystems: a microcosm study.  
485 *Environ Sci Nano* **2016**, *3* (4), 830–838.
- 486 (22) Larue, C.; Laurette, J.; Herlin-Boime, N.; Khodja, H.; Fayard, B.; Flank, A.-M.; ...  
487 Carriere, M. Accumulation, translocation and impact of TiO<sub>2</sub> nanoparticles in wheat (*Triticum*  
488 *aestivum* spp.): influence of diameter and crystal phase. *Sci. Total Environ.* **2012**, *431*, 197–208.
- 489 (23) Debelle, F. The *Medicago truncatula* genome. In *Biological Nitrogen Fixation*; de  
490 Bruijn, F. J. John Wiley & Sons: 2015; pp 787–789.
- 491 (24) Tesfaye, M.; Samac, D. A.; Vance, C. P. Insights into symbiotic nitrogen fixation in  
492 *Medicago truncatula*. *MPMI* **2006**, *19* (3), 330–341. doi: 10.1094/MPMI-19-0330.
- 493 (25) Simonin, M.; Richaume, A.; Guyonnet, J. P.; Dubost, A.; Martins, J. M. F.; Pommier, T.  
494 Titanium dioxide nanoparticles strongly impact soil microbial function by affecting archaeal  
495 nitrifiers. *Scientific Reports* **2016**, *6*(1), 33643. <https://doi.org/10.1038/srep33643>
- 496 (26) de Vauflery, A.; Coeurdassier, M.; Pandard, P.; Scheifler, R.; Lovy, C.; Crini, N.;  
497 Badot, P.-M. How terrestrial snails can be used in risk assessment of soils. *Environ. Toxicol.*  
498 *Chem.* **2006**, *25*(3), 797–806.
- 499 (27) Mouchet, F.; Landois, P.; Puech, P.; Pinelli, E.; Flahaut, E.; Gauthier L. CNT  
500 ecotoxicity in amphibians: assessment of multi walled carbon nanotubes (MWNT) and

- 501 comparison with double walled carbon nanotubes (DWNT). Special focus environmental toxicity  
502 of nanoparticles. *Nanomedicine* **2010**, *5*(6), 963–974.
- 503 (28) Lagier, L.; Mouchet, F.; Laplanche, C.; Mottier, A.; Cadarsi, S.; Evariste, L.; Sarrieu, C.;  
504 Lonchambon, P.; Pinelli, E.; Flahaut, E.; Gauthier, L. Surface area of carbon-based nanoparticles  
505 prevails on dispersion for growth inhibition in amphibians. *Carbon* **2017**, *119*, 72–81.  
506 <https://doi.org/10.1016/j.carbon.2017.04.016>
- 507 (29) Mouchet, F.; Gauthier, L.; Mailhes, C.; Jourdain, M.J.; Ferrier, V.; Triffault, G.; Devaux,  
508 A. Biomonitoring of the genotoxic potential of aqueous extracts of soils and bottom ash resulting  
509 from municipal solid waste incineration, using the comet and micronucleus tests on amphibian  
510 (*Xenopus laevis*) larvae and bacterial assays (Mutatox® and Ames tests). *Sci. Total Env.* **2006**,  
511 *355* (1-3): 232–246.
- 512 (30) Nieuwkoop, P.D.; Faber, J. Normal tables of *Xenopus laevis* (Daudin). Amsterdam 7  
513 North Holland Publ.; **2006**.
- 514 (31) ISO 21427-1. Qualité de l'eau—Évaluation de la génotoxicité par le mesurage de  
515 l'induction de micronoyaux—Partie 1: Évaluation de la génotoxicité à l'aide de larves  
516 d'amphibiens Water quality—Evaluation of genotoxicity by measurement of the induction of  
517 micronuclei—Part 1: Evaluation of genotoxicity using amphibian larvae. **2006**.
- 518 (32) R Core Team. R: a language and environment for statistical computing. R foundation for  
519 Statistical Computing; 2015.
- 520 (33) Fox, J.; Weisberg, S. An {R} Companion to Applied Regression, 2nd ed.; Thousand  
521 Oaks CA: Sage, 2011.
- 522 (34) Graves, S.; Piepho, H.-P.; Selzer, L. Visualizations of Paired Comparisons; 2015.
- 523 (35) Giraudoux, P. pgirmess: Data Analysis in Ecology; 2017.

- 524 (36) Lenth, R. V. Least-Squares Means: The R Package lsmeans. *J. Stat. Softw.* **2016**, *69* (1),  
525 1–33.
- 526 (37) Rafique, R.; Arshad, M.; Khokhar, M. F.; Qazi, I. A., Hamza, A.; Virk, N. Growth  
527 Response of Wheat to Titania Nanoparticles Application. *NUST Journal of Engineering Sciences*  
528 **2015**, *7* (1), 42–46.
- 529 (38) Prajapati, K; Modi, H.A. The importance of potassium in plant growth—a review. *An*  
530 *Online International Journal* **2012**, *1* (2-3), 177–186.
- 531 (39) Maathuis, F. J. M. Sodium in plants: perception, signalling, and regulation of sodium  
532 fluxes. *Journal of Experimental Botany* **2014**, *65* (3), 849–858.
- 533 (40) Pi, Z.; Stevanato, P.; Yv, L. H.; Geng, G.; Guo, X. L.; Yang, Y., ... Kong, X. S. Effects  
534 of potassium deficiency and replacement of potassium by sodium on sugar beet plants. *Russ.*  
535 *J. Plant Physiol.* **2014**, *61* (2), 224–230.
- 536 (41) Servin, A.; Morales, M. I.; Castillo-Michel, H.; Hernandez-Viezcas, J.; Munoz, B.; Zhao,  
537 L.; Nunez, J. E.; Peralta-Videa, J. R.; Gardea-Torresday, J. L. Synchrotron verification of TiO<sub>2</sub>  
538 accumulation in cucumber fruit : a possible pathway of TiO<sub>2</sub> nanoparticle transfer from soil into  
539 the food chain. *Environ Sci Technol.* **2013**, *47* (20), 11592–11598.
- 540 (42) Rafique, R.; Zahra, Z.; Virk, N.; Shahid, M.; Pinelli, E.; Kallerhoff, J.; Park, T. N.;  
541 Arshad, M. Data on rhizosphere pH, phosphorus uptake and wheat growth responses upon TiO<sub>2</sub>  
542 nanoparticles application. *Data in Brief.* **2018**, *17*, 890–896.
- 543 (43) Imran, M.; Gurmani, Z. A. Role of macro and micro nutrients in the plant growth and  
544 development. *Science Technology and Development (Pakistan)*, **2011**. Retrieved from  
545 <http://agris.fao.org/agris-search/search.do?recordID=PK2012000898>
- 546 (44) Ge, Y.; Priester, J. H.; Van De Werfhorst, L. C.; Schimel, J. P.; Holden, P. A. Potential

- 547 mechanisms and environmental controls of TiO<sub>2</sub> nanoparticle effects on soil bacterial  
548 communities. *Environmental Science and Technology*. **2013**, *47*, 14411–14417.
- 549 (45) Ge, Y.; Schimel, J. P.; Holden, P. A. Identification of soil bacteria susceptible to TiO<sub>2</sub>  
550 and ZnO. *Appl. Environ. Microbiol.* **2012**, *78*, 6749–6758. doi:10.1128/AEM.00941-12
- 551 (46) Simonin, M.; Martins, J. M. F.; Le Roux, X.; Uzu, G., Calas, A.; Richaume, A. Toxicity  
552 of TiO<sub>2</sub>nanoparticles on soil nitrification at environmentally relevant concentrations: lack of  
553 classical dose-response relationships. *Nanotoxicology* **2017**, *11* (2), 247–255.
- 554 (47) Bissett, A.; Brown, M. V.; Siciliano, S. D.; Thrall, P. H. Microbial community responses  
555 to anthropogenically induced environmental change: towards a systems approach. *Ecol. Lett.*  
556 **2013**, *16* Suppl 1, 128–139.
- 557 (48) van der Heijden, M. G. A.; Bardgett, R. D.; van Straalen, N. M. The unseen majority: soil  
558 microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol. Lett.* **2008**,  
559 *11* (3), 296–310.
- 560 (49) Scheifler, R.; de Vaufleury, A.; Coeurdassier, M.; Crini, N.; Badot, P.-M. Transfer of Cd,  
561 Cu, Ni, Pb, and Zn in a soil-plant-invertebrate food chain: a microcosm study. *Environ. Toxicol.*  
562 *Chem.* **2006**, *25* (3), 815–822.
- 563 (50) Eybe, T.; Audinot, J.-N.; Udelhoven, T.; Lentzen, E.; El Adib, B.; Ziebel, J., ... Bohn, T.  
564 Determination of oral uptake and biodistribution of platinum and chromium by the garden snail  
565 (*Helix aspersa*) employing nano-secondary ion mass-spectrometry. *Chemosphere*. **2013**, *90* (6),  
566 1829–1838.
- 567 (51) Gogos, A.; Moll, J.; Klingenfuss, F.; van der Heijden, M.; Irin, F.; Green, M. J.; Zenobi,  
568 R.; Bucheli, T. D. Vertical transport and plant uptake of nanoparticles in a soil mesocosm  
569 experiment. *J Nanobiotechnology* 2016, *14* (40). doi: <https://doi.org/10.1186/s12951-016-0191-z>

- 570 (52) Chenon, P.; Gauthier, L.; Loubières, P.; Séverac, A.; Delpoux, M. Evaluation of the  
571 genotoxic and teratogenic potential of a municipal sludge and sludge-amended soil using the  
572 amphibian *Xenopus laevis* and the tobacco: *Nicotiana tabacum* L. var. *xanthi* Dulieu.  
573 *Sci. Total Environ.* **2003**, *301*, 139–150.
- 574 (53) Mouchet, F.; Cren, S.; Cunienq, C.; Deydier, E.; Guilet, R.; Gauthier, L. Assessment of  
575 lead ecotoxicity in water using the amphibian larvae (*Xenopus laevis*) and preliminary study of  
576 its immobilization in meat and bone meal combustion residues. *Biometals.* **2007**, *20*(2), 113–127.
- 577 (54) Bekaert, C.; Rast, C.; Ferrier, V.; Bispo, A.; Jourdain, M. J.; Vasseur, P. Toxicity and  
578 genotoxicity of industrial soils polluted by polycyclic aromatic hydrocarbons (PAHs). *Org.*  
579 *Geochem.* **1999**, *30*, 953–962.
- 580 (55) Bacchetta, R.; Tremolada, P.; Di Benedetto, C.; Santo, N.; Fascio, U.; Chirico, G., . . .  
581 Mantecca, P. Does carbon nanopowder threaten amphibian development? *Carbon N Y*, **2012**, *50*  
582 (12), 4607–4618.
- 583 (56) Bacchetta, R.; Santo, N.; Fascio, U.; Moschini, E.; Freddi, S.; Chirico, G.; Camatini,  
584 M.; Mantecca, P. Nano-sized CuO, TiO<sub>2</sub> and ZnO affect *Xenopus laevis* development.  
585 *Nanotoxicology.* **2012**, *6*, 381–398.
- 586 (57) Zhu, X.; Zhu, L.; Duan, Z.; Qi, R.; Li, Y.; Lang, Y. Comparative toxicity of several metal  
587 oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. *J*  
588 *Environ Sci Health A Tox Hazard Subst Environ Eng* **2008**, *43* (3), 278–284.
- 589 (58) Federici, G.; Shaw, B. J.; Handy, R. D. Toxicity of titanium dioxide nanoparticles to  
590 rainbow trout (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological  
591 effects. *Aquat. Toxicol.* **2007**, *84*(4), 415–430.