



**HAL**  
open science

# Combined study of titanium dioxide nanoparticle transport and toxicity on microbial nitrifying communities under single and repeated exposures in soil columns

Marie Simonin, Jean M. F. Martins, Gaëlle Uzu, Erwann Vince, Agnès Richaume

## ► To cite this version:

Marie Simonin, Jean M. F. Martins, Gaëlle Uzu, Erwann Vince, Agnès Richaume. Combined study of titanium dioxide nanoparticle transport and toxicity on microbial nitrifying communities under single and repeated exposures in soil columns. *Environmental Science and Technology*, 2016, 50 (19), pp.10693-10699. 10.1021/acs.est.6b02415 . hal-01604452

**HAL Id: hal-01604452**

**<https://hal.science/hal-01604452>**

Submitted on 15 Oct 2021

**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.

1 **A combined study of TiO<sub>2</sub> nanoparticle transport and toxicity on microbial**  
2 **nitrifying communities under single and repeated exposures in soil columns**

3 Marie SIMONIN<sup>1,2,3,4†</sup>, Jean M.F. MARTINS<sup>4,5</sup>, Gaëlle UZU<sup>4,6</sup>, Erwann VINCE<sup>4,5</sup> and Agnès  
4 RICHAUME<sup>1,2,3\*</sup>

5 <sup>1</sup> Université de Lyon, Lyon, France

6 <sup>2</sup> Université Claude Bernard Lyon 1, Villeurbanne, France

7 <sup>3</sup> CNRS, UMR 5557, Microbial Ecology Lab, Université Lyon 1, Villeurbanne, France

8 <sup>4</sup> Université Grenoble Alpes, LTHE UMR 5564, Grenoble F-38000, France

9 <sup>5</sup> CNRS, LTHE, F-38000 Grenoble, France

10 <sup>6</sup> IRD, LTHE, F-38000 Grenoble, France

11

12 \*Correspondence to: [agnes.richaume@univ-lyon1.fr](mailto:agnes.richaume@univ-lyon1.fr)

13 †Present address: Department of Biology, Duke University, Durham, NC, USA; Center for the  
14 Environmental Implications of Nanotechnology, Duke University, Durham, NC, United States.

15

16

17 Révision Soumise le 25/07/2016

18

19

20

21

22

23

24 **ABSTRACT**

25 Soils are exposed to nanoparticles (NPs) due to their increasing use in many commercial  
26 products. Adverse effects of NPs on soil microorganisms have been reported in several  
27 ecotoxicological studies using microcosms. Although repeated exposures are more likely to  
28 occur in soils, most of these previous studies were performed as a single exposure to NPs.  
29 Contrarily to single contamination, the study of multiple NP contaminations in soils requires  
30 the use of specialized setups. Using a soil column experiment, we compared the influence of  
31 single and repeated exposures (one, two or three exposures that resulted in the same final  
32 concentration) on the transport of titanium dioxide NPs (TiO<sub>2</sub>-NPs) through soil and the effect  
33 of these different exposure scenarios on the abundance and activity of soil nitrifying microbial  
34 communities after a 2-month incubation period. The transport of TiO<sub>2</sub>-NPs was very limited  
35 under both single and repeated exposures and was highest for the lowest concentration  
36 injected during the first application. Significant decreases in nitrification activity and  
37 ammonia-oxidizing archaea and bacteria populations were observed only for the repeated  
38 exposure scenario (three TiO<sub>2</sub>-NP contaminations). These results show that under repeated  
39 exposures, the transport of TiO<sub>2</sub>-NPs to deep soil layers and groundwater is limited and that a  
40 chronic TiO<sub>2</sub>-NPs contamination is more harmful for the soil microbiological functioning than  
41 a single exposure.

42

43 **Key-words:** TiO<sub>2</sub> nanomaterials, chronic and acute exposure, transport, microbial  
44 ecotoxicology, nitrification.

45

## 46 INTRODUCTION

47 In recent years, several studies have highlighted deleterious effects of metal-oxide  
48 nanoparticles (NPs) on soil (micro)organisms and, consequently, on soil functioning and  
49 fertility<sup>1-3</sup>. In most of these studies, soils were exposed to single homogeneous applications  
50 of NPs into microcosms<sup>3</sup>. However, NPs are more likely to be released onto the soil surface  
51 through repeated applications of biosolids, irrigation or through the use of nanofertilizers or  
52 nanopesticides<sup>4-6</sup> and may be leached to further depths through soil porosity<sup>7</sup>. Therefore in  
53 contaminated soils NPs are heterogeneously distributed, especially along vertical soil profiles<sup>8</sup>.  
54 This heterogeneous distribution of NPs is controlled by the soil physicochemical properties  
55 that are known to influence the mobility of colloids according to the colloidal filtration  
56 theory<sup>9-11</sup> and also by NP properties, such as size and/or surface charge<sup>7</sup>.

57 To date, only a few studies were performed on repeated or chronic NP exposure in soil<sup>12</sup>. The  
58 lack of studies dealing with chronic NP toxicity in soils is mainly due to the experimental  
59 difficulties associated with executing repeated exposures in microcosm experiments.  
60 Implementing more appropriate experimental designs that reflect realistic environmental  
61 conditions and consider the distribution of NPs according to soil depth are therefore required.

62 The aim of this work was to compare the effect of single and repeated NP applications on: (i)  
63 the transport of TiO<sub>2</sub>-NPs through a soil profile, and (ii) their toxicity to the soil microbial  
64 communities by focusing on disruption of the nitrification process which is a critical step in  
65 the nitrogen cycle. Nitrification is a crucial microbiological process for soil fertility controlled  
66 by the activity of both of ammonia- and nitrite-oxidizers that convert ammonium to nitrate.  
67 This key step of the nitrogen cycle is known to be very sensitive to environmental stressors,  
68 especially chronic disturbances<sup>12-14</sup>. Therefore, the abundance of ammonia-oxidizing archaea

69 and bacteria (AOA and AOB, respectively) was selected for monitoring soil toxicity in this study  
70 because this endpoint has been proposed as relevant ecological indicators for soil health<sup>15,16</sup>.  
71 TiO<sub>2</sub>-NPs are the most produced NPs and it has been predicted that they may represent 50 %  
72 of all engineered NPs retrieved in soil, especially because of the repeated application of  
73 biosolids on agricultural soils<sup>17,18</sup>. TiO<sub>2</sub>-NPs are known to be toxic for microbes, even in the  
74 absence of light like in conditions encountered in soils, due to a depolarization and loss of  
75 membrane integrity translating in a cell osmotic stress<sup>19,20</sup>

76 Our experimental design, based on saturated soil columns, enabled the study of the fate and  
77 impact of both single and repeated TiO<sub>2</sub>-NP exposures in soil under more realistic exposure  
78 conditions than what can be achieved with classical microcosm experiments. In the context of  
79 short-term lab-scale experiment, soil columns were contaminated using three exposure  
80 scenarios (one, two, or three applications) resulting in the same total TiO<sub>2</sub>-NP concentrations  
81 applied to soil columns (372 mg NPs kg<sup>-1</sup> dry soil). After the last NP application, four layers of  
82 the contaminated soil columns corresponding to different depths were collected and  
83 incubated separately for 2 months to evaluate the impact of TiO<sub>2</sub>-NP exposure on the  
84 nitrification process along the soil profile. The nitrification enzyme activity (NEA) and the  
85 abundance of AOA and AOB were measured.

86

## 87 **MATERIALS AND METHODS**

### 88 **Soil**

89 The upper 20 cm layer of a silty-clay soil (Cambisol, WRB, 2006) under a permanent pasture  
90 was collected at Commarin (Côte d'Or, France). After soil sampling, roots and plant litter were

91 manually removed. The soil was sieved at 2 mm and homogenized before storage at 4 °C. The  
92 main soil characteristics were analyzed by the Laboratoire d'analyse des sols (INRA Arras,  
93 France) following NF ISO protocols: sand, 10 %; loam, 51 %; clay, 39 %; pH 7.7; organic matter  
94 (OM), 7.9%; cation exchange capacity (CEC), 20.1 cmol kg<sup>-1</sup>; water holding capacity (WHC), 51  
95 %; and ionic strength, 1.37 mM.

## 96 **TiO<sub>2</sub> nanoparticles**

97 Uncoated titanium dioxide nanoparticles (mixture of anatase (80%) and rutile (20%) crystal  
98 structure) were purchased from Sigma Aldrich (St Louis, USA) with at least 99.5 % purity. The  
99 mean primary particle size of the TiO<sub>2</sub>-NPs was 28.7 (± 7.1) nm in powder as measured with a  
100 ZEISS Ultra 55 scanning electron microscopy–field emission gun (SEM-FEG) and energy  
101 dispersive spectroscopy (EDS) with a SDD detector (BRUKERAXS-30 mm<sup>2</sup>).

102 The size and zeta potential of the TiO<sub>2</sub>-NPs were characterized using Dynamic Light Scattering  
103 (DLS) in the soil solution used as the background solution in column experiments. The soil  
104 solution was prepared following the protocol described by Simonin *et al.*<sup>21</sup>. Briefly, 10 g of soil  
105 dispersed in 50 mL of ultrapure water were shaken for 30 min at 180 rpm and 20 °C in a  
106 refrigerated incubator shaker (New Brunswick – Eppendorf, Hamburg, Germany). The  
107 solutions were then centrifuged for 20 min at 8000g and 20 °C (Centrifuge 5804R, Eppendorf,  
108 Hamburg, Germany) to eliminate particles larger than 20 nm according to Stokes' law. The  
109 supernatants were collected and stored at 4 °C. The resulting soil solution had a pH of 7.1, an  
110 ionic strength of 1.6 mM and a dissolved organic carbon (DOC) concentration of 8.9 mg L<sup>-1</sup>.  
111 Measurements were performed in triplicate with three suspensions of TiO<sub>2</sub>-NPs (50, 25 and  
112 16.7 mg L<sup>-1</sup>) after dispersion by ultrasonication (Sonication bath, Bioblock Scientific) for 5 min

113 to ensure the suspension homogeneity. For each sample, the mean of three measurements  
114 was recorded.

### 115 **Exposure of soil in columns to single and repeated TiO<sub>2</sub>-NP applications**

116 Three TiO<sub>2</sub>-NP suspensions were prepared in soil solution for each exposure scenario: 50 mg  
117 L<sup>-1</sup> for the single exposure experiment, 25 mg L<sup>-1</sup> for the two-exposures experiment, and 16.7  
118 mg L<sup>-1</sup> for the three-exposures experiment. For the three exposure scenarios, the soil columns  
119 received the same net amount of NPs, corresponding to a final concentration of 372 mg kg<sup>-1</sup>  
120 dry soil, which is high compared to the predicted TiO<sub>2</sub> concentration (1.2 mg kg<sup>-1</sup> year<sup>-1</sup>) in  
121 European soils<sup>18</sup>. The choice of a high input concentration of TiO<sub>2</sub>-NPs was made to ensure a  
122 titanium concentration in the column effluents that was high enough to be distinguished from  
123 the background concentration. Indeed, soils naturally contain very high concentrations of Ti<sup>22</sup>  
124 (in average 4000-5000 mg kg<sup>-1</sup> and 4520 ± 233 mg Ti kg<sup>-1</sup> dry soil in the studied soil). After  
125 saturation and leaching with 100 mL of the soil solution, the column effluents before NP-  
126 spiking exhibited an average concentration of 0.33 ± 0.1 mg Ti L<sup>-1</sup>. Since we observed that  
127 TiO<sub>2</sub>-NP aggregation and surface charge were significantly different in water and soil  
128 solution<sup>21</sup>, the suspensions of TiO<sub>2</sub>-NPs were prepared in soil solution to achieve a more  
129 realistic assessment of the fate and impact of NPs in the soil.

130 Soil exposures to NPs were performed in 1 x 10 cm glass columns (C10/10, GE Healthcare),  
131 homogeneously packed with soil (8 g equivalent dry soil), at 28 °C in the dark (see Figure S1  
132 for schematic figure of the column experimental set up). Flow adaptors (AC 10, GE Healthcare)  
133 were adjusted on the top of the columns to ensure constant and similar soil heights during  
134 experiments. At the beginning of the experiment, soil columns were saturated and leached  
135 bottom-up with 100 mL of the pristine soil solution to reduce and stabilize the background Ti

136 concentration in the effluents. During the entire transport experiment, the solutions were  
137 injected in the column using a peristaltic pump at a flow rate of  $0.1 \text{ mL min}^{-1}$ , which  
138 corresponds to a Darcy flow of  $0.15 \text{ cm h}^{-1}$ . After this first step of saturation/leaching, 5 pore  
139 volumes (PV) (each pore volume was 6.7 mL) of the  $\text{TiO}_2$ -NPs suspension (either 50, 25 or 16.7  
140  $\text{mg L}^{-1}$ ) were injected and then 10 PV of the soil solution free of  $\text{TiO}_2$ -NPs were immediately  
141 injected in the columns.

142 The single exposure experiment was performed as a one-time injection of  $50 \text{ mg L}^{-1}$   $\text{TiO}_2$ -NPs  
143 into six saturated soil columns. Six control columns received the same PV of the soil solution  
144 free of  $\text{TiO}_2$ -NPs.

145 Repeated exposure experiments were conducted as two or three applications of  $\text{TiO}_2$ -NPs with  
146 25 and  $16.7 \text{ mg L}^{-1}$ , respectively in 6 columns for each treatment. Six additional control  
147 columns were used for each type of exposure as mentioned above. The successive  
148 applications of NPs were separated by a 15-day delay during which columns were maintained  
149 horizontally at  $28 \text{ }^\circ\text{C}$  in the dark at constant moisture.

#### 150 **Transport of $\text{TiO}_2$ -NPs in soil columns**

151 For each exposure treatment, effluents from a randomly chosen column were continuously  
152 sampled into 15 mL centrifuge tubes (1 mL sampled in 10 minutes) using a fraction collector  
153 (Gilson, Minipulse 3). Ti concentrations in the spiking suspensions ( $C_0$ ) and in the effluents (C)  
154 were determined using a microwave assisted (Novawave, SCP Science) strong acid extraction  
155 method (hydrofluoric acid + nitric acid) and were used to establish dimensionless  $\text{TiO}_2$   
156 breakthrough curves ( $C/C_0$ ). Ti concentrations were measured by inductively coupled plasma  
157 - optical emission spectrophotometry (ICP-OES; Varian 700-ES, Varian Inc. Scientific  
158 Instruments, Palo Alto, USA). Control columns leached with NP-free soil solution enabled the



159 determination of the background concentration of titanium in the effluents. In order to ensure  
160 the quality of the titanium measurements, a certified reference material was measured along  
161 with our samples: Sandy Loam 10, RTC-CRM027, lot HC027 certified reference material from  
162 Sigma-Aldrich. The concentrations found were within 95–102 % of the certified values for all  
163 measured elements.

164 Consistently with Nickel *et al.*<sup>23</sup>, TiO<sub>2</sub>-NP concentrations retained in the different soil layers  
165 after contamination could not be accurately measured due to the naturally high titanium  
166 content of the soil (4520 mg Ti kg<sup>-1</sup> dry soil) compared to the TiO<sub>2</sub>-NP input concentration (372  
167 mg kg<sup>-1</sup>). Indeed, the standard error of the soil total Ti content measurements (233 mg kg<sup>-1</sup>  
168 dry soil) was too close to the TiO<sub>2</sub>-NP concentration added to the soil to expect reliable values  
169 of TiO<sub>2</sub>-NPs concentration in the different soil layers. Thus, our estimation of the final TiO<sub>2</sub>-NP  
170 concentration retained in soil is based on the measured Ti concentrations (mass balance) in  
171 the column effluents and not a direct measurement of the Ti concentration in soil.

172

### 173 **Impact of TiO<sub>2</sub>-NPs on the nitrification process along the soil profile**

174 The impact of TiO<sub>2</sub>-NPs on soil nitrification activity and ammonia-oxidizing microorganisms  
175 abundance as a function of soil depth was studied 2 months after the last exposure to NPs.  
176 Immediately after the last exposure, soil columns were sliced in four layers according to the  
177 distance from the inlet (0–2 cm, 2–4 cm, 4–6 cm and 6–8 cm). Each slice was then placed into  
178 60 mL glass flasks and incubated at constant moisture for 2 months at 28 °C in the dark. This  
179 experimental design resulted in 144 samples: 6 column treatments (three exposure scenarios  
180 and three control treatments) x 4 depths x 6 replicates. At the end of the incubation time, 1.5  
181 g of soil (equivalent dry weight, dw) were immediately used for the measurements of

182 nitrification enzyme activity (NEA) and 0.5 g of soil were stored at -20 °C before DNA  
183 extraction.

184

#### 185 **DNA extraction and abundance of ammonia-oxidizers**

186 DNA was extracted from 0.5 g of frozen soil using the Fast DNA<sup>®</sup> spin Kit for soil (MP  
187 Biomedicals, Solon, OH, USA), following the manufacturer's instructions and then DNA  
188 concentrations were determined using the Qubit dsDNA BR Assay (Invitrogen).

189 The abundance of ammonia-oxidizers (AOA and AOB) was measured by quantitative PCR using  
190 a Lightcycler 480 (Roche Diagnostics, Meylan, France). The primers and thermal cycling  
191 conditions used are described in Table 1.

192 Ammonia monooxygenase (*amoA*) gene abundance measurements for AOA and AOB  
193 quantification were performed in a final reaction volume of 20 µL and contained (final  
194 concentrations) 0.5 µM of each primer for the bacterial *amoA* or 0.75 µM of CrenamoA616r  
195 and 1 µM of CrenamoA23f for the archaeal *amoA*, 2 % bovine serum albumin (BSA), 1X of  
196 QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France) and 10 ng of soil DNA  
197 extract or 10<sup>7</sup>–10<sup>2</sup> gene copies number of an in-house plasmid containing cloned bacterial  
198 (*Nitrosomonas europaea*, GenBank accession number:L08050) and archaeal (54d9 fosmid  
199 fragment<sup>24</sup>) *amoA* genes. Melting curve analysis confirmed the specificity of amplification of  
200 the two genes.

201

202

203 **Nitrification enzyme activity**

204 NEA was determined according to the protocol described by Dassonville *et al.*<sup>25</sup>. Sub-samples  
205 of fresh soil (1.5 g equivalent dry weight) were incubated with 3 mL of a solution of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
206 in order to reach nitrogen-as-ammonia (N-NH<sub>4</sub><sup>+</sup>) concentrations of 50 µg g<sup>-1</sup> dry soil. Distilled  
207 water was added to each sample to achieve 12 mL of total liquid volume in glass plasma flasks.  
208 The flasks were sealed with Parafilm® and incubated at 28 °C under 180 rpm constant  
209 agitation. During the incubation, 1 mL of soil slurry was sampled at 2 h, 4 h, 6 h, 8 h and 10 h,  
210 filtered (0.2 µm pore size) and transferred in vials stored at -20 °C. The analysis of NO<sub>3</sub><sup>-</sup>  
211 concentrations was performed by ionic chromatography (DX120, Dionex, Salt Lake City, USA)  
212 equipped with a 4 mm × 250 mm column (IonPac AS9 HC). NEA was expressed as µg nitrogen-  
213 as-nitrate (N-NO<sub>3</sub><sup>-</sup>) h<sup>-1</sup> g<sup>-1</sup> dry soil.

214

215 **Statistical analysis**

216 All results are presented as means (± standard error). A two-way analysis of variance (ANOVA)  
217 and *post-hoc* Tukey's honest significant difference (HSD) were performed to test the effect of  
218 TiO<sub>2</sub>-NP exposures and soil depth on the nitrifying activity and ammonia-oxidizer abundance  
219 for each type of exposure scenario separately. Data were log-transformed prior to analysis  
220 when necessary to ensure conformity with the assumptions of normality and homogeneity of  
221 variances. T-tests were conducted to compare the aggregation and zeta potential of TiO<sub>2</sub>-NPs  
222 in the different spiking suspensions. All statistical analyses were carried out using the R  
223 statistical software 2.13.2<sup>26</sup>.

224

## 225 **RESULTS**

### 226 **Characteristics of TiO<sub>2</sub>-NPs in the spiking soil solutions**

227 TiO<sub>2</sub>-NPs were characterized in the suspensions used to contaminate soil columns under the  
228 three exposure scenarios. The aggregation of TiO<sub>2</sub>-NPs increased with the concentration  
229 (Table 2). At the lowest concentrations applied three times successively in soil columns (16.7  
230 mg L<sup>-1</sup>), the average TiO<sub>2</sub>-NP hydrodynamic diameter was 111.2 (± 10.1) nm, while at the  
231 highest concentration used for the single application (50 mg L<sup>-1</sup>), the hydrodynamic diameter  
232 was 153.9 (± 9.97) nm.

233 The surface charge of TiO<sub>2</sub>-NPs assessed through zeta potential measurements  
234 (electrophoretic mobility) were extremely close for the three suspension concentrations  
235 (-15.4 to -16.6 mV; Table 2), even if it was significantly lower in the 25 mg L<sup>-1</sup> suspension (Table  
236 2).

### 237 **Effect of TiO<sub>2</sub>-NP concentration and of the number of applications on their mobility in soil**

238 The experimental design enabled the study of the influence of TiO<sub>2</sub>-NPs concentration on their  
239 transport through soil after single exposures at 16.7, 25 or 50 mg L<sup>-1</sup> and after repeated  
240 exposures consisting of two or three applications of NPs in suspensions containing 25 and 16.7  
241 mg L<sup>-1</sup>, respectively (Table 3).

242 The mobility of TiO<sub>2</sub>-NPs decreased with increasing TiO<sub>2</sub>-NP concentration (Table 3). For an  
243 injected concentration of 16.7 mg L<sup>-1</sup>, 10.66 % of TiO<sub>2</sub>-NPs were recovered in the effluents,  
244 whereas only 4.95 and 2.19 % were recovered when applying the suspensions containing 25  
245 and 50 mg L<sup>-1</sup>, respectively.

246 In the columns that received two applications of NPs, the mobility of TiO<sub>2</sub>-NPs was greatly  
247 reduced compared to the mobility measured during the first application (Table 3). Only 0.9 %  
248 of the added TiO<sub>2</sub>-NPs were recovered in the effluents for both 25 and 16.7 mg L<sup>-1</sup> exposure  
249 concentrations (Table 3). For the third NP application at 16.7 mg L<sup>-1</sup>, the mobility of TiO<sub>2</sub>-NPs  
250 was almost negligible (0.2%; Table 3).

251 We calculated the final TiO<sub>2</sub>-NP concentration retained in the column on the basis of the mass  
252 recovery calculations in the effluents after background subtraction (Table 3). Although the  
253 same theoretical TiO<sub>2</sub>-NPs concentration of 372 mg kg<sup>-1</sup> was applied to the soil, the final NPs  
254 retained concentration differed in the three exposure scenarios (Table 3). The highest final  
255 concentration of TiO<sub>2</sub>-NPs (364 mg kg<sup>-1</sup>) was measured in the columns exposed to a single  
256 application at 50 mg L<sup>-1</sup>. In the columns receiving two applications at 25 mg L<sup>-1</sup>, the final TiO<sub>2</sub>-  
257 NPs concentration was estimated at 350 mg kg<sup>-1</sup>, which corresponds to a decrease of 6 %  
258 compared to the single exposure. After three applications at 16.7 mg L<sup>-1</sup>, only 328 mg kg<sup>-1</sup> of  
259 TiO<sub>2</sub>-NPs were retained in the soil, which corresponds to a decrease of 12 % and 6 %,  
260 compared to the single and dual exposure scenarios, respectively.

### 261 **Effect of single and repeated NP contaminations on nitrification enzyme activity and** 262 **ammonia-oxidizer abundance**

263 After two months of incubation following the last application of NPs to soil columns, NEA, AOA  
264 and AOB abundances were measured. When considering the whole soil column (i.e. the four  
265 soil layers), the single exposure (i.e. single application at 50 mg L<sup>-1</sup>) resulted in a significant  
266 increase of the AOA abundance (Figure 1; Table 4) but no effect was observed on AOB  
267 abundance or NEA (Figure 1; Table 4). After two successive NP applications at 25 mg L<sup>-1</sup>,  
268 neither the NEA nor the ammonia-oxidizer abundances were affected by TiO<sub>2</sub>-NPs

269 contamination (Figure 1, Table 4). In contrast, NEA, AOA and AOB abundance were  
270 significantly decreased after three repeated applications of TiO<sub>2</sub>-NPs at 16.7 mg L<sup>-1</sup> (Figure 1;  
271 Table 4).

272 A significant effect of soil depth on NEA was only observed after two and three exposures ( $P$   
273 = 0.02 and  $P$  = 0.003, respectively; Table 4). After two soil exposures to NPs, the NEA differed  
274 significantly according to soil depth, especially at 2–4 cm (Figure 2). After three exposures,  
275 NEA was significantly more reduced by TiO<sub>2</sub>-NPs in the first 2 cm than in the other soil layers  
276 (Figure 2). No significant statistical interaction between TiO<sub>2</sub>-NPs contamination and soil  
277 depth was found (Table 4).

278

## 279 **DISCUSSION**

### 280 **Consequences of single and repeated exposures on TiO<sub>2</sub>-NPs transport in soil**

281 Our experimental design based on soil columns dynamic contamination enabled coupled  
282 mobility and toxicity studies of TiO<sub>2</sub>-NPs under single and repeated exposures at different  
283 input concentrations. The results showed that the transport of TiO<sub>2</sub>-NPs is low (90 to 99% of  
284 retention) and influenced by the concentration injected in the soil columns. After a single  
285 application, TiO<sub>2</sub>-NP retention in soil increased with increasing injected concentrations.  
286 Similar behavior of silica nanoparticles has been reported in sand columns by Vitorge *et al.*<sup>27</sup>,  
287 who showed that NPs mobility increases at low concentration regimes. These results can be  
288 explained by the increase of TiO<sub>2</sub>-NP aggregation with increasing concentration, which can  
289 favor the filtration, deposition and straining of these colloids<sup>7,28</sup>. Although the significant  
290 difference of TiO<sub>2</sub>-NP aggregation between the three concentrations tested in soil solution (42

291 nm maximum, 28% of the highest hydrodynamic diameter measured) supports this  
292 hypothesis, the involvement of other factors such as heteroaggregation kinetics cannot be  
293 ruled out.

294 Previous studies on the mobility of uncoated TiO<sub>2</sub>-NPs in soil reported contrasting results.  
295 Nickel et al. (2015)<sup>23</sup> observed no transport using an input concentration of 1 g TiO<sub>2</sub>-NPs L<sup>-1</sup>,  
296 while significant transport was recorded in different soils exhibiting low clay contents and  
297 ionic strengths with several applied concentrations of TiO<sub>2</sub>-NPs<sup>9</sup>. Here, we show that although  
298 low, the transport of TiO<sub>2</sub>-NPs can occur after a single exposure despite the fine texture of the  
299 studied soil (39 % clay and 51 % loam), especially at the lowest concentration regime, which  
300 is more likely to be observed in natural soils. In the case of an accidental spill for which high  
301 NPs concentrations can be expected, the mobility of TiO<sub>2</sub>-NPs will be likely very low, in  
302 agreement with a classical blocking mechanism typically observed at high colloidal  
303 concentrations<sup>27,28</sup>

304 For repeated exposure in soil columns, an extremely low mobility of TiO<sub>2</sub>-NPs was observed  
305 (< 1%) and no concentration effect was observed contrarily to what was observed with the  
306 single exposures. The distribution of TiO<sub>2</sub>-NPs along the soil profile could not be measured  
307 without specific labeling<sup>23</sup>. However, these results observed in only 8 cm long columns suggest  
308 that in soils repeatedly exposed to TiO<sub>2</sub>-NPs, these contaminants would accumulate in the top  
309 soil, where, coincidentally, most of the biological activity occurs. In these conditions, it is likely  
310 that NPs transport to deeper soil layers and groundwater is limited. In order to confirm this  
311 assumption future studies are needed to assess TiO<sub>2</sub>-NPs transport in longer soil columns and  
312 in undisturbed soil cores.

313

314 **Deleterious effects on nitrification after repeated exposure**

315 Two months after the last application of TiO<sub>2</sub>-NPs to the soil columns, significant decreases in  
316 NEA and ammonia-oxidizer abundances were observed only in soil samples exposed three  
317 times to the lowest TiO<sub>2</sub>-NPs concentration (16.7 mg L<sup>-1</sup>). In this case, the final TiO<sub>2</sub>-NPs  
318 concentration in soil was slightly lower than in the two other exposure scenarios. Surprisingly,  
319 we have observed an increase of AOA abundance in the single exposure scenario but without  
320 significant changes on NEA. A possible explanation is that a shift occurred in the AOA  
321 community and that AOA populations with lower nitrifying activity were stimulated in these  
322 conditions. We show in this short-term experiment that soil repeated exposure to TiO<sub>2</sub>-NPs  
323 has a more deleterious impact on soil microbial community and soil functioning than a single  
324 exposure. This result highlights that the nitrifying population did not become more resistant  
325 and resilient to TiO<sub>2</sub>-NPs after multiple exposures. On the contrary, the nitrification process  
326 and ammonia-oxidizers became more sensitive, probably because organisms stressed by the  
327 previous exposures to NPs had less energy to cope with additional disturbances and/or  
328 because of a decrease or a shift of soil microbial diversity<sup>29</sup>. It is well known that a microbial  
329 community can become tolerant to various stressors over time through the selection of more  
330 tolerant populations and/or the dispersal of specific resistances *via* mobile genetic  
331 elements<sup>14,29,30</sup>. Chronic exposures to pollutants occurring over a longer term will likely have  
332 less consequences on nitrification because of potential microbial community adaptation over  
333 time, as it was observed by Mertens *et al.*<sup>31</sup> after 2 years of exposure to zinc pollution. To gain  
334 a better knowledge of NP impact on soil functioning under chronic contamination, it would be  
335 necessary to study the effects on microbial activity, abundance and diversity after each  
336 exposure and before each new exposure, in order to monitor the resistance and resilience of  
337 the microbial community in the long term. This approach could lead to new insights into



338 sensitive and tolerant microbial species among nitrifiers and thus on TiO<sub>2</sub>-NP toxicity and  
339 adaptation mechanisms in soil.

340 After three NP applications, it is likely that most of TiO<sub>2</sub>-NPs were retained close to the inlet  
341 of the column through filtration and/or straining. Although a TiO<sub>2</sub> concentration gradient can  
342 be expected along the soil profile according to the filtration theory<sup>32</sup>, no significant effect of  
343 depth on ammonia-oxidizer abundance was observed. Nitrification was significantly more  
344 diminished in the 0–2 cm and 6–8 cm layers than in the middle 2–4 and 4–6 cm soil layers.  
345 With common soluble pollutants, the general assumption is that the highest concentrations  
346 induce the most deleterious effects. In the case of NPs, concentration effects are more  
347 complex because of aggregation processes and thus the reactivity, bioavailability and toxicity  
348 of these contaminants can vary in function of the spiked concentration<sup>21,33–35</sup>. Such changes  
349 in NPs aggregation with concentration are likely to induce nonlinear dose-effect  
350 relationships<sup>36</sup>. Our results suggest that microbial communities may be affected by TiO<sub>2</sub>-NPs  
351 at the depths of preferential NP accumulation (top of the soil column) but also at deeper  
352 depths likely reached by NPs at lower concentration (bottom of the soil column).

353 The multidisciplinary approach developed in this study enabled the comparison of the impact  
354 of single and repeated TiO<sub>2</sub>-NPs exposures in water-saturated soil columns. The transport of  
355 TiO<sub>2</sub>-NPs in the studied silty-clay soil was evidenced but was limited (over 90% retention) for  
356 both single and repeated applications in 8 cm long columns. TiO<sub>2</sub>-NPs mobility was also shown  
357 to increase at low NPs concentrations indicating an aggregation-controlled process.  
358 Considering these results, the transport of TiO<sub>2</sub>-NPs to deeper soil layers or groundwater  
359 appears limited. Although the repeat exposures resulted in lower final TiO<sub>2</sub>-NP concentrations  
360 in soil, the impact was greater on the soil microbial community and on soil functioning (NEA)

361 compared to the single exposure condition. Future studies should address the fate and effects  
362 of TiO<sub>2</sub>-NPs chronically added to soils in biosolid amendments, as it is one of the most relevant  
363 routes by which NPs enter the soil environment<sup>37</sup>. Chronic exposure in short and long term  
364 experiments representing realistic scenarios of soil exposure to NPs are urgently needed to  
365 study the resistance and resilience of soil microbial communities to these emerging  
366 contaminants and their consequences on soil fertility.

367

368

#### 369 **TABLE CAPTIONS**

370

371 **Table 1** PCR primers and thermal cycling conditions used for quantification of ammonia-  
372 oxidizer abundances

373

374 **Table 2** Size and zeta potential of TiO<sub>2</sub>-NPs in soil solution for the 3 tested concentrations  
375 measured with a Nano ZS (Malvern). Means and standard errors are presented (n = 3). Values  
376 labeled with the same letter do not differ at  $P < 0.05$ .

377

378 **Table 3** TiO<sub>2</sub> relative mass recoveries in column effluents for the three applied NPs  
379 concentrations in the different exposure scenarios (one, two, or three applications). The final  
380 TiO<sub>2</sub>-NP concentration retained in the column was calculated from the relative mass recovery  
381 of TiO<sub>2</sub>-NPs in the effluents. The presented values have been corrected by the Ti background  
382 concentration in the effluents measured before NP application (0.33 mg Ti L<sup>-1</sup>).

383

384 **Table 4** P-values from an ANOVA analysis of the effects of TiO<sub>2</sub>-NPs, soil depth and their  
385 interaction on nitrification enzyme activity, AOA and AOB abundances for each exposure  
386 scenario. Values in bold are significant at  $P < 0.05$ .

387

#### 388 **FIGURE CAPTIONS**

389 **Figure 1** Effect of TiO<sub>2</sub>-NPs on nitrification enzyme activity (NEA), AOA and AOB abundances  
390 in whole columns (average of the 4 soil depths). Data are expressed as the percentage of  
391 change relative to the control treatment for each exposure. Standard errors are presented (n  
392 = 6). Significant effects are indicated (\*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ).

393

394 **Figure 2** Effect of TiO<sub>2</sub>-NPs on nitrification enzyme activity (NEA) measured in the four soil  
395 column layers (depths 0-2 cm; 2-4 cm; 4-6 cm; 6-8 cm) after one, two or three soil exposures.  
396 Data are expressed as percentage of change from the control treatment for each exposure  
397 condition. Standard errors are presented (n = 6). For each exposure scenario, bars labeled with  
398 the same letter do not differ at  $P < 0.05$ .

399

#### 400 **ACKNOWLEDGMENTS**

401 Marie Simonin was supported by a Ph.D grant from Rhône-Alpes Region – ARC  
402 Environnement. This work was funded by a grant from the French National Program EC2CO  
403 Microbien of CNRS. The authors thank the technical assistance of Sungeun Lee. Nitrification  
404 enzyme activity measurements were performed at the AME platform (Microbial Ecology

405 UMR5557-USC1364, Lyon) and quantitative PCR at the DTAMB platform (IFR 41, University  
406 Lyon 1). LTHE is part of the Labex OSUG@2020 (ANR10 LABX56).

407

## 408 REFERENCES

- 409 (1) Dinesh, R.; Anandaraj, M.; Srinivasan, V.; Hamza, S. Engineered nanoparticles in the soil  
410 and their potential implications to microbial activity. *Geoderma* **2012**, *173–174*, 19–27.
- 411 (2) Pan, B.; Xing, B. Applications and implications of manufactured nanoparticles in soils: a  
412 review. *Eur. J. Soil Sci.* **2012**, *63* (4), 437–456.
- 413 (3) Simonin, M.; Richaume, A. Impact of engineered nanoparticles on the activity,  
414 abundance, and diversity of soil microbial communities: a review. *Environ. Sci. Pollut.*  
415 *Res.* **2015**, 1–14.
- 416 (4) Brar, S. K.; Verma, M.; Tyagi, R. D.; Surampalli, R. Y. Engineered nanoparticles in  
417 wastewater and wastewater sludge – Evidence and impacts. *Waste Manag.* **2010**, *30*  
418 (3), 504–520.
- 419 (5) Liu, R.; Lal, R. Potentials of engineered nanoparticles as fertilizers for increasing  
420 agronomic productions. *Sci. Total Environ.* **2015**, *514*, 131–139.
- 421 (6) Servin, A.; Elmer, W.; Mukherjee, A.; Torre-Roche, R. D. la; Hamdi, H.; White, J. C.;  
422 Bindraban, P.; Dimkpa, C. A review of the use of engineered nanomaterials to suppress  
423 plant disease and enhance crop yield. *J. Nanoparticle Res.* **2015**, *17* (2), 1–21.
- 424 (7) Cornelis, G.; Hund-Rinke, K.; Kuhlbusch, T.; Brink, N. van den; Nickel, C. Fate and  
425 Bioavailability of Engineered Nanoparticles in Soils: A Review. *Crit. Rev. Environ. Sci.*  
426 *Technol.* **2014**, *44* (24), 2720–2764.
- 427 (8) Navarro, D. A.; Banerjee, S.; Watson, D. F.; Aga, D. S. Differences in Soil Mobility and  
428 Degradability between Water-Dispersible CdSe and CdSe/ZnS Quantum Dots. *Environ.*  
429 *Sci. Technol.* **2011**, *45* (15), 6343–6349.
- 430 (9) Fang, J.; Shan, X.; Wen, B.; Lin, J.; Owens, G. Stability of titania nanoparticles in soil  
431 suspensions and transport in saturated homogeneous soil columns. *Environmental*  
432 *Pollution* **2009**, *157*, 1101–1109.
- 433 (10) Martins, J. M. F.; Majdalani, S.; Vitorge, E.; Desaunay, A.; Navel, A.; Guiné, V.; Daïan, J.  
434 F.; Vince, E.; Denis, H.; Gaudet, J. P. Role of macropore flow in the transport of  
435 *Escherichia coli* cells in undisturbed cores of a brown leached soil. *Environ. Sci. Process.*  
436 *Impacts* **2013**, *15* (2), 347–356.
- 437 (11) Vitorge, E.; Szenknect, S.; Martins, J. M. F.; Barthès, V.; Auger, A.; Renard, O.; Gaudet,  
438 J.-P. Comparison of three labeled silica nanoparticles used as tracers in transport  
439 experiments in porous media. Part I: Syntheses and characterizations. *Environ. Pollut.*  
440 **2014**, *184*, 605–612.
- 441 (12) Dalzell, D. J. B.; Alte, S.; Aspichueta, E.; de la Sota, A.; Etxebarria, J.; Gutierrez, M.;  
442 Hoffmann, C. C.; Sales, D.; Obst, U.; Christofi, N. A comparison of five rapid direct toxicity  
443 assessment methods to determine toxicity of pollutants to activated sludge.  
444 *Chemosphere* **2002**, *47* (5), 535–545.

- 445 (13) Broos, K.; Mertens, J.; Smolders, E. Toxicity of heavy metals in soil assessed with various  
446 soil microbial and plant growth assays: A comparative study. *Environ. Toxicol. Chem.*  
447 **2005**, *24* (3), 634–640.
- 448 (14) Bissett, A.; Brown, M. V.; Siciliano, S. D.; Thrall, P. H. Microbial community responses to  
449 anthropogenically induced environmental change: towards a systems approach. *Ecol.*  
450 *Lett.* **2013**, *16*, 128–139.
- 451 (15) Schloter, M.; Dilly, O.; Munch, J. C. Indicators for evaluating soil quality. *Agric. Ecosyst.*  
452 *Environ.* **2003**, *98* (1–3), 255–262.
- 453 (16) Wessén, E.; Hallin, S. Abundance of archaeal and bacterial ammonia oxidizers – Possible  
454 bioindicator for soil monitoring. *Ecol. Indic.* **2011**, *11* (6), 1696–1698.
- 455 (17) Keller, A. A.; McFerran, S.; Lazareva, A.; Suh, S. Global life cycle releases of engineered  
456 nanomaterials. *J. Nanoparticle Res.* **2013**, *15* (6), 1–17.
- 457 (18) Sun, T. Y.; Gottschalk, F.; Hungerbühler, K.; Nowack, B. Comprehensive probabilistic  
458 modelling of environmental emissions of engineered nanomaterials. *Environ. Pollut.*  
459 **2014**, *185*, 69–76.
- 460 (19) Sohm, B.; Immel, F.; Bauda, P.; Pagnout, C. Insight into the primary mode of action of  
461 TiO<sub>2</sub> nanoparticles on *Escherichia coli* in the dark. *Proteomics* **2015**, *15* (1), 98–113.
- 462 (20) Erdem, A.; Metzler, D.; Cha, D. K.; Huang, C. P. The short-term toxic effects of TiO<sub>2</sub>  
463 nanoparticles toward bacteria through viability, cellular respiration, and lipid  
464 peroxidation. *Environ. Sci. Pollut. Res.* **2015**, *22* (22), 17917–17924.
- 465 (21) Simonin, M.; Guyonnet, J. P.; Martins, J. M. F.; Ginot, M.; Richaume, A. Influence of soil  
466 properties on the toxicity of TiO<sub>2</sub> nanoparticles on carbon mineralization and bacterial  
467 abundance. *J. Hazard. Mater.* **2015**, *283*, 529–535.
- 468 (22) Aubert, H.; Pinta, M. *Trace Elements in Soils*; Elsevier, 1980.
- 469 (23) Nickel, C.; Gabsch, S.; Hellack, B.; Nogowski, A.; Babick, F.; Stintz, M.; Kuhlbusch, T. A.  
470 Mobility of coated and uncoated TiO<sub>2</sub> nanomaterials in soil columns—Applicability of  
471 the tests methods of OECD TG 312 and 106 for nanomaterials. *J. Environ. Manage.* **2015**,  
472 *157*, 230–237.
- 473 (24) Treusch, A. H.; Leininger, S.; Kletzin, A.; Schuster, S. C.; Klenk, H.-P.; Schleper, C. Novel  
474 genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated  
475 mesophilic crenarchaeota in nitrogen cycling. *Environ. Microbiol.* **2005**, *7* (12), 1985–  
476 1995.
- 477 (25) Dassonville, N.; Guillaumaud, N.; Piola, F.; Meerts, P.; Poly, F. Niche construction by the  
478 invasive Asian knotweeds (species complex *Fallopia*): impact on activity, abundance and  
479 community structure of denitrifiers and nitrifiers. *Biol. Invasions* **2011**, *13* (5), 1115–  
480 1133.
- 481 (26) R Core Team. R: A language and environment for statistical computing. R Foundation  
482 for Statistical Computing. Vienna, Austria 2015.
- 483 (27) Vitorge, E.; Szenknect, S.; Martins, J. M. F.; Gaudet, J.-P. Size- and concentration-  
484 dependent deposition of fluorescent silica colloids in saturated sand columns: transport  
485 experiments and modeling. *Environ. Sci. Process. Impacts* **2013**, *15* (8), 1590–1600.
- 486 (28) Solovitch, N.; Labille, J.; Rose, J.; Chaurand, P.; Borschneck, D.; Wiesner, M. R.; Bottero,  
487 J.-Y. Concurrent Aggregation and Deposition of TiO<sub>2</sub> Nanoparticles in a Sandy Porous  
488 Media. *Environ. Sci. Technol.* **2010**, *44* (13), 4897–4902.
- 489 (29) Griffiths, B. S.; Philippot, L. Insights into the resistance and resilience of the soil  
490 microbial community. *FEMS Microbiol. Rev.* **2013**, *37* (2), 112–129.

- 491 (30) Allison, S. D.; Martiny, J. B. H. Resistance, resilience, and redundancy in microbial  
492 communities. *Proc. Natl. Acad. Sci.* **2008**, *105* (Supplement 1), 11512–11519.
- 493 (31) Mertens, J.; Broos, K.; Wakelin, S. A.; Kowalchuk, G. A.; Springael, D.; Smolders, E.  
494 Bacteria, not archaea, restore nitrification in a zinc-contaminated soil. *ISME J.* **2009**, *3*  
495 (8), 916–923.
- 496 (32) Huber, N.; Baumann, T.; Niessner, R. Assessment of Colloid Filtration in Natural Porous  
497 Media by Filtration Theory. *Environ. Sci. Technol.* **2000**, *34*, 3774–3779.
- 498 (33) Choi, O. K.; Hu, Z. Q. Nitrification inhibition by silver nanoparticles. *Water Sci. Technol.*  
499 **2009**, *59* (9), 1699.
- 500 (34) Simon-Deckers, A.; Loo, S.; Mayne-L’hermite, M.; Herlin-Boime, N.; Menguy, N.;  
501 Reynaud, C.; Gouget, B.; Carrière, M. Size-, Composition- and Shape-Dependent  
502 Toxicological Impact of Metal Oxide Nanoparticles and Carbon Nanotubes toward  
503 Bacteria. *Environ. Sci. Technol.* **2009**, *43* (21), 8423–8429.
- 504 (35) Menard, A.; Drobne, D.; Jemec, A. Ecotoxicity of nanosized TiO<sub>2</sub>. Review of in vivo data.  
505 *Environ. Pollut.* **2011**, *159* (3), 677–684.
- 506 (36) Bernhardt, E. S.; Colman, B. P.; Hochella, M. F.; Cardinale, B. J.; Nisbet, R. M.;  
507 Richardson, C. J.; Yin, L. An ecological perspective on nanomaterial impacts in the  
508 environment. *J. Environ. Qual.* **2010**, *39* (6), 1954–1965.
- 509 (37) Pradas del Real, A. E.; Castillo-Michel, H.; Kaegi, R.; Sinnet, B.; Magnin, V.; Findling, N.;  
510 Villanova, J.; Carrière, M.; Santaella, C.; Fernández-Martínez, A.; et al. Fate of Ag-NPs in  
511 Sewage Sludge after Application on Agricultural Soils. *Environ. Sci. Technol.* **2016**, *50* (4),  
512 1759–1768.
- 513

514