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# Influence of soil type on TiO<sub>2</sub> nanoparticle fate in an agro-ecosystem

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## Abstract:

Nanoparticles (NPs) and in particular TiO<sub>2</sub>-NPs are increasingly included in commercial goods leading to their accumulation in sewage sludge which is spread on agricultural soils as fertilizers in many countries. Crop plants are thus a very likely point of entry for NPs in the food chain up to humans. So far, soil influence on NP fate has been under-investigated. In this article, we studied the partitioning of TiO<sub>2</sub>-NPs between soil and soil leachate, their uptake and biotransformation in wheat seedlings and their impact on plant development after exposure on 4 different types of soil with different characteristics: soil texture (from sandy to clayey), soil pH, cationic exchange capacity, organic matter content. Results suggest that a NP contamination occurring on agricultural soils will mainly lead to NP accumulation in soil (increase of Ti concentration up to 302 % in sand) but to low to negligible transfer to soil leachate and plant shoot. In our experimental conditions, no sign of acute phytotoxicity has been detected (growth, biomass, chlorophyll content). Clay content above 6% together with organic matter content above 1.5% lead to translocation factor from soil to plant leaves below 2.5% (*i.e.* below

30 13 mg Ti.kg<sup>-1</sup> dry leaves). Taken together, our results suggest low risk of crop contamination in an agro-  
31 ecosystem.

32

33 Keywords: agriculture, bioavailability, ecotoxicology, food safety, plant, transfer

34 **1. Introduction:**

35 For more than a decade, **nanotechnologies** have attracted the interest of industrials and scientists  
36 throughout the world. The manipulation of matter at the nanometer scale opens up new possibilities  
37 in many different fields, such as industry, medicine and sustainable development. Nanotechnologies  
38 imply the use of a huge amount of nanoparticles (NPs), *i.e.* particles with at least one dimension below  
39 100 nm. The new properties exhibited by NPs are also a source of questions about a potentially  
40 different behavior and toxicity.

41 The number of **commercial goods** available on the market and (officially) containing NPs has  
42 increased from 54 in 2005 to 2850 in 2016 in all fields of everyday life (cosmetics, food containers,  
43 sporting goods, personal care, medicine, construction, agriculture)<sup>1,2</sup>. **TiO<sub>2</sub>-NPs** are one of the most  
44 used NPs, appearing in about 25% of the products according to nano-databases<sup>1,2</sup>. One can estimate a  
45 yearly production of TiO<sub>2</sub>-NPs comprised between 1 000 and 10 000 tons<sup>3</sup>. Under their nanometric  
46 form, they are mainly used for their photocatalytic properties for instance in construction materials  
47 (self-cleaning materials) or for their UV-adsorbing properties in cosmetics (sunscreens)<sup>3</sup>. Besides, they  
48 can be found in small proportions in food additives<sup>4,5</sup>.

49

50 This expansion of the use of TiO<sub>2</sub>-NPs is inevitably leading to an increased **dissemination in the**  
51 **environment**. The release may be accidental, during the production or transport of NPs, or chronic,  
52 during the life cycle of products containing NPs. It has been shown that, TiO<sub>2</sub>-NPs contained in certain  
53 textiles and paints are detached from their matrix during laundry or weathering and finally end up in  
54 wastewater treatment plants<sup>7-10</sup> and in particular in sewage sludge, where they can reach a  
55 concentration of 2 g.kg<sup>-1</sup><sup>11</sup>. Moreover, the spreading of sewage sludge as soil fertilizer is a common  
56 practice in agriculture with quantities reaching 3 tons (dry weight)/ha/year and is regulated for  
57 instance in the US, in Australia and in Europe for sludge heavy metal content but nothing about NPs  
58 <sup>12-15</sup>. This leads to the dissemination of TiO<sub>2</sub>-NPs in agricultural soils. In addition, the expected  
59 environmental concentrations could be much higher locally with the intentional introduction of NPs in

60 the environment. For instance, thanks to their unique UV properties, TiO<sub>2</sub>-NPs are used for purification  
61 purposes in both soil and water<sup>16-19</sup>. Another critical case is the use of nanopesticides<sup>20,21</sup>. Indeed, TiO<sub>2</sub>-  
62 NPs are used in crop protection products for some years in order to optimize the product efficiency  
63 and then to eventually reduce the amount of inputs into the environment (sustainable development).  
64 The introduction of these products on the market is also expected to increase in the coming years<sup>22-</sup>  
65 <sup>25</sup>.

66 Until very recently, most of the NP risk assessment studies have been carried out on a single  
67 organism in simplified conditions (hydroponics), exposed to high concentrations of NPs during short  
68 time, simulating acute contaminations<sup>26,27</sup>. Over the last years, studies related to the fate of NPs in the  
69 environment evidenced a trend towards more realistic exposure scenario: soil exposure, longer  
70 exposure period and lower NP concentrations<sup>26,27</sup>.

71 Studying the fate of TiO<sub>2</sub>-NPs in soil is challenging. Indeed, soil is a very complex matrix. Moreover,  
72 the contribution of TiO<sub>2</sub> engineered nanomaterials is challenging to measure in the soil because of the  
73 high geogenic Ti background ( $\approx 0.6\%$  of the terrestrial crust). Accordingly, to date, only modelling  
74 studies can help approximate the amount of engineered TiO<sub>2</sub>-NPs that is ending up in the environment.  
75 The last article estimation forecasts the presence of 61 mg of TiO<sub>2</sub>.kg<sup>-1</sup> of soil amended with sewage  
76 sludge<sup>11</sup>, making **agricultural soil** the main sink for nanomaterials in the environment and crop plants  
77 a privileged point of entry for NPs in the food chain up to humans.

78 Currently, knowledge about the interactions of NPs with soil is very limited. Once NPs reach the  
79 soil, they can be either physically retained or chemically adsorbed onto the surface of soil particles<sup>27</sup>.  
80 These interactions could mitigate NP phytotoxicity and bioavailability<sup>27</sup>, meaning that a same  
81 contamination could lead to very distinct consequences according to soil type. The literature published  
82 on plants exposed in soils suggested a very limited transfer of NPs to aerial parts. For a better risk  
83 assessment, it is thus of primary importance to understand how soil characteristics control NP fate in  
84 a crop system. For this purpose we exposed wheat, a widely grown crop over the world, to TiO<sub>2</sub>-NPs

85 (0, 100, 500 mg.kg<sup>-1</sup>) in 4 different types of soil already containing geogenic Ti at different  
 86 concentrations. Ti quantification in soil, soil leachates and wheat leaves was assessed. Ti distribution  
 87 in roots and leaves and speciation in leaves were also determined using large scale research facilities.  
 88 Finally NP phytotoxicity was evaluated through plant development parameters: height, fresh and dry  
 89 weights and chlorophyll content.

90

## 91 **2. Material and Methods**

92

### 93 **2.1. Experimental set-up**

94 TiO<sub>2</sub>-NPs with a nominal diameter of 25 nm and a crystalline phase mainly anatase (89%) were used  
 95 (see Figure S1 for more details).

96 A sand and 3 agricultural soils provided by Lufa Speyer (Germany) were chosen with different  
 97 characteristics (Table 1): texture ranging from sand to clayey loam, organic carbon content from 0 to  
 98 2.09%, cation exchange capacity (CEC) from 0 to 10 meq/100 g and pH from 5.1 to 7.2.

99

	<b>Sand</b>	<b>Silty sand</b>	<b>Loamy sand</b>	<b>Clayey loam</b>
<b>Particle size (%)</b>				
<0.002 mm	0.0 ± 0.0	2.9 ± 0.8	6.4 ± 0.9	41.7 ± 1.3
0.002-0.05 mm	0.0 ± 0.0	9.1 ± 1.4	11.6 ± 0.7	36.1 ± 2.2
0.05 – 2 mm	100.0 ± 0.0	88.0 ± 1.0	82.0 ± 0.7	22.2 ± 1.5
<b>Water holding capacity</b>				
g/100 g	25.3 ± 2.8	31.8 ± 3.0	46.5 ± 6.0	39.6 ± 5.0
<b>Weight per Volume</b>				
g/1000 mL	1756 ± 6	1430 ± 57	1220 ± 78	1330 ± 96
<b>Organic Carbon</b>				
%	0.0 ± 0.0	0.74 ± 0.14	2.09 ± 0.40	1.69 ± 0.17

pH value				
0.01M CaCl <sub>2</sub>	6.3 ± 0.0	5.1 ± 0.5	5.5 ± 0.1	7.2 ± 0.1
Cation Exchange Capacity				
Meq/100g	<2	4.0 ± 1.0	10.0 ± 0.5	22.0 ± 6.0
Leachates				
mL	10.6 ± 0.1 <sup>c</sup>	9.1 ± 0.2 <sup>bc</sup>	8.0 ± 0.3 <sup>ab</sup>	6.3 ± 0.6 <sup>a</sup>

100

101 *Table 1. Soil characteristics (soil provided by Lufa Speyer - Germany: silty sand: soil 2.1, loamy sand:*  
102 *soil 2.2, clayey loam: soil 6S). Characteristics were provided by the supplier. For leachates, different*  
103 *lowercase letters indicate significant differences (p<0.05) among all treatments.*

104

105 Exposure concentrations of TiO<sub>2</sub>-NPs represented a realistic condition (100 mg.kg<sup>-1</sup>) and a higher and  
106 plausible concentration after several years of sewage sludge spreading (500 mg.kg<sup>-1</sup>). TiO<sub>2</sub>-NP  
107 suspensions (100 or 500 mg.L<sup>-1</sup> and ultrapure water as control) were mixed with soil with a ratio of 1:1  
108 (w:w) for 12 hours on a shaker (200 rpm) in containers wrapped with aluminum foil. This mixing  
109 strategy was chosen to ensure the most homogeneous matrix as possible for plant exposure. After this  
110 step, the mixture was poured in a folded standard filter paper (Fisherbrand, Dia/Size 330 mm) and  
111 separated in soil leachates and soil (after 8h of settling). After weighting, soil leachates were  
112 evaporated and prepared for Ti quantification to determine the Ti mobility.

113 6 individual replicates for each type of soils (4) and each level of contamination (3) were set-up with  
114 each a wheat seed (*Triticum aestivum* L., cv. Courtaud). All experiments were carried out in a growth  
115 chamber under controlled conditions: day/night photoperiod (16/8 h), 100 μmol/m<sup>2</sup>/s, day/night  
116 temperature, (24/20±1 °C) and day/night relative humidity (70/75%). Leaf height and chlorophyll  
117 content (determined using a SPAD-502 chlorophyll meter, Minolta Camera Co., Japan) were assessed  
118 every day for 3 weeks until harvest. At the end of the experiment fresh leaf weight and dry leaf weight  
119 after 48h in an oven at 70°C were recorded.

120

## 121 **2.2. Bulk Ti quantification by ICP-AES**

122 Different tests have been performed to ensure a proper acidic digestion of TiO<sub>2</sub>-NPs which represents  
123 a critical step to avoid underestimation of Ti concentration in the samples. Several digestion protocols  
124 adapted from the literature have been compared (Figure S2A) with different acid cocktails,  
125 temperatures and evaporation steps. The best digestion yield was obtained with a protocol adapted  
126 from Mariet *et al.* (2008) with a mix of HNO<sub>3</sub>, NH<sub>4</sub>F, HClO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub> with several evaporation steps at  
127 100 °C in a DigiPrep<sup>®</sup> system (see Supporting Information for more details)<sup>28</sup>. The yield was about 88%  
128 and reproducible. This test demonstrates that the digestion protocol needs to be re-optimized for each  
129 TiO<sub>2</sub>-NP. Indeed, in one of our previous experiments using 4 nm anatase TiO<sub>2</sub>-NPs, the protocol from  
130 Mariet *et al.* was not efficient <sup>29</sup>. The linearity of the digestion was also assessed with increasing TiO<sub>2</sub>-  
131 NP concentrations (Figure S2B). Finally the stability of the solution after digestion over a month was  
132 monitored (Figure S2C). After digestion, samples (soils, leachates, plant shoots) were analyzed using  
133 Inductively Coupled Plasma – Mass Spectrometry (ICP-AES IRIS, Thermo Scientific). Quality of sample  
134 preparation was checked with blank samples containing only chemicals and with reference samples  
135 (tomato leaves, NIST RM 1573a) and permitted to validate sample preparation and ICP analysis (Figure  
136 S2bis, Table S1).

137

## 138 **2.3. Spatially resolved Ti semi-quantification by μPIXE/RBS**

139 A combination of micro-particle induced X-ray emission (μPIXE) and Rutherford Backscattered  
140 spectroscopy (RBS) has been used for Ti semi-quantification. μPIXE/RBS quantification of Ti was chosen  
141 as a spatially resolved technique to avoid mixing the Ti signal coming from NPs adsorbed at the surface  
142 of roots with Ti really absorbed inside the root tissues.

143 Wheat roots were thoroughly washed with deionized water to take off NPs lightly bound to the surface  
144 and immersed in a droplet of resin (Tissue Teck Sakura<sup>®</sup>) to be immediately cryo-fixed by plunging the



145 sample in isopentane cooled with liquid nitrogen. Samples were then cut in thin cross-sections (50  $\mu\text{m}$ )  
146 using a cryo-microtome (Leica) and finally freeze-dried (48h,  $-52^\circ\text{C}$ , 0.01 mbars).  
147 Freeze-dried sections were analyzed at the nuclear microprobe available at the Atomic Energy  
148 Commission (CEA) Center of Saclay (France) with a proton source of 3 MeV, a beam focused to 2.5  $\mu\text{m}$   
149 and a current intensity of 500 pA. Data processing was performed using Rismin<sup>30</sup> software to define  
150 regions of interest and extract spectra, and SIMNRA<sup>31</sup> and GUPIX<sup>32</sup> codes to reduce RBS and PIXE  
151 data, respectively. The Ti/Ca ratio was used as a Ti enrichment indicator as Ca is an abundant  
152 endogenous element.

153

#### 154 **2.4. Ti distribution and speciation by $\mu\text{XRF}$ and $\mu\text{XANES}$**

155 Samples were prepared as described previously for nuclear microprobe experiments (section 2.3.).  
156 Synchrotron experiments were carried out on LUCIA beamline at SOLEIL (Saint Aubain, France)<sup>33</sup>. Micro  
157 X-ray fluorescence ( $\mu\text{XRF}$ ) maps were recorded above the Ti-K edge (5.1 keV) with a step size of 5  $\mu\text{m}$   
158 and a dwell time of 2000 ms.  $\mu\text{XRF}$  data were fitted using PyMCA software<sup>34</sup>.

159 Ti-K edge (4.5 to 5.5 keV energy range, 0.5 eV step) micro X-ray absorption near edge spectroscopy  
160 ( $\mu\text{XANES}$ ) spectra were recorded in regions of interest of the fluorescence maps. XANES spectra on  
161 reference compounds were recorded during previous experiments<sup>29,35</sup>. For the fitting, 3 groups of  
162 references were created: anatase, rutile and others (containing Ti-oxysulfate, amorphous Ti oxide and  
163 Ti acetate). XANES data treatment was performed using Athena software<sup>36</sup> as previously<sup>29,35</sup>.

164

#### 165 **2.5. Statistical analysis**

166 Data were checked for normality (Shapiro test) and homoscedasticity (Bartlett or Levene's tests). They  
167 were compared using two-way ANOVA, with soil type and  $\text{TiO}_2$ -NP concentration as main effects, and  
168 the interaction (soil type x NP concentration). If the whole set of data did not follow a normal  
169 distribution even after transformation, Student T test, Wilcoxon test, one-way ANOVA or Kruskal–  
170 Wallis test were performed on subset of data (according to soil type or NP concentration). Post hoc

171 tests (Tukey's HSD or Dunn's test) were applied to detect differences between groups of samples.  
172 Differences were considered significant when  $P < 0.05$ . All statistical analyses are gathered in Table  
173 S21.

174 To study the global influence of  $\text{TiO}_2$ -NPs on plant elemental content, MANOVA based on Pillai's trace  
175 test was used on the whole set of micro- and macronutrients measured by ICP-AES.

176 All statistical analyses were performed using the R statistical software (version 3.1.3)<sup>37</sup>, with *car*<sup>38</sup>,  
177 *multcompView*<sup>39</sup>, *pgirmess*<sup>40</sup> and *lsmeans*<sup>41</sup> packages.

178

179

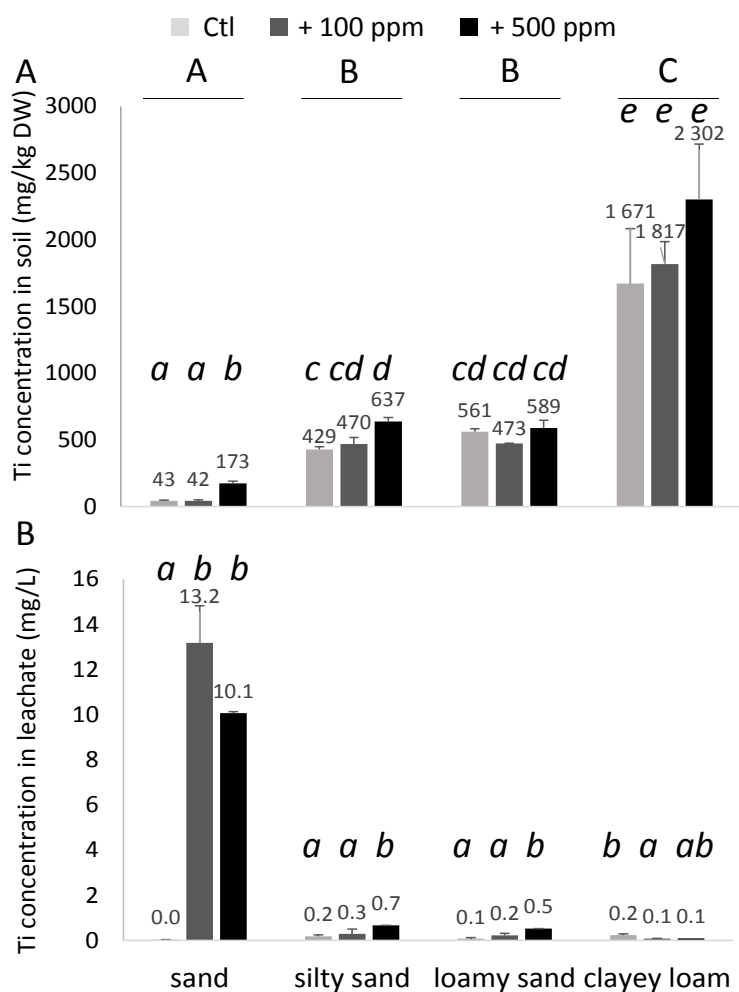
### 180 **3. Results and discussion**

#### 181 **3.1. Ti partitioning between soil and soil leachates**

182 As stated earlier, Ti is a geogenic element of the earth crust. As such, the 4 natural soils (control  
183 condition) contained Ti concentrations ranging from 43 to 1671  $\text{mg}\cdot\text{kg}^{-1}$  with sand having the lowest  
184 concentration followed by the group of silty and loamy sands and finally the clayey loam being the  
185 richest in Ti ( $P < 0.0001$ , Figure 1A). Ti concentration in natural soils was positively correlated with the  
186 fraction of soil particles in the 0.002 to 0.005 mm size range ( $r^2 = 0.9997$ , Table 1) and to a lesser extent  
187 to the fraction  $< 0.002$  mm ( $r^2 = 0.9618$ ). After addition of 100  $\text{mg}\cdot\text{kg}^{-1}$  of  $\text{TiO}_2$ -NPs, the Ti concentration  
188 in soils did not significantly increase in any soil. After addition of 500  $\text{mg}\cdot\text{kg}^{-1}$  of  $\text{TiO}_2$ -NPs, the increase  
189 in Ti was only significant for sand and silty sand ( $P < 0.0001$ ), *i.e.* soils with the lowest clay fraction.

190 Those results can be confronted with Ti concentrations in soil leachates (Figure 1B). In natural soils, Ti  
191 concentrations in the leachates after 12h mixing were respectively 0.03, 0.18, 0.07 and 0.23  $\text{mg}\cdot\text{L}^{-1}$  for  
192 sand, silty sand, loamy sand and clayey loam. After addition of 100  $\text{mg}\cdot\text{kg}^{-1}$  of  $\text{TiO}_2$ -NPs, only the sand  
193 leachate had a significantly higher Ti concentration (13.18  $\text{mg}\cdot\text{L}^{-1}$ ,  $P = 0.0379$ ) with a more than 400 fold  
194 increase in comparison with the control sand. This is also the soil in which the leachate volume  
195 recovered was the highest (Table 1): 71% of the 15 mL of the contamination suspension was collected  
196 in the leaching compartment. This fraction was decreased to 42% in the case of the clayey loam soil.

197 Crossing this result with Ti concentration in soil, one can conclude that most of the TiO<sub>2</sub>-NP suspension  
 198 added in the 100 mg.kg<sup>-1</sup> condition went through the sand column and ended up in the soil leachate.  
 199 After addition of 500 mg.kg<sup>-1</sup> of TiO<sub>2</sub>-NPs, Ti concentration in soil leachate was significantly increased  
 200 as compared to the control for all soils except for the clayey loam soil ( $P=0.0019$  for silty sand and  $P=$   
 201  $0.0031$  for loamy sand).



202  
 203 *Figure 1. Ti concentration in the soil system: soil (A) and soil leachate (B) determined by ICP-AES.*  
 204 *Different lowercase letters indicate significant differences ( $p<0.05$ ) among all treatments in A or among*  
 205 *treatment within a soil type in B. Capital letters in A highlight significant differences among soil types.*  
 206 *( $n=6$ ).*

207

208 If a contamination occurs on a sand, most TiO<sub>2</sub>-NPs will likely go through the soil and reach the aquifers.  
209 Only a small fraction will remain in the soil if a high dose (500 mg.kg<sup>-1</sup>) of NPs is applied. In the silty  
210 sand, Ti increase in both soil and soil leachate was only detectable for the highest concentration added.  
211 For the loamy sand, the high Ti natural background (> 500 mg.kg<sup>-1</sup>) shadowed the addition of NPs in  
212 the soil and Ti increase was visible only in the leachate at the highest NP concentration added. Finally,  
213 in the clayey loam, all added Ti was retained in the soil but did not increase significantly Ti  
214 concentration also because of the very high geogenic Ti level. Depth column studies would be needed  
215 to confirm these results about NP mobility and gain a deeper understanding.

216 Soils with coarser texture, lower organic carbon content as well as lower CEC (sand and silty sand) were  
217 the ones in which NPs displayed the highest availability. This result is in agreement with previous  
218 literature<sup>42-45</sup>. Authors suggested that soils with a higher clay content have also smaller pore size and  
219 larger surface area which increase both electrostatic interactions and physical straining explaining the  
220 lower transport of NPs to soil leachates. Likewise, Cu-NP translocation was observed to be low in soils  
221 enriched in organic matter and high in clay<sup>46</sup>. Another important point that could be considered is the  
222 interaction of NPs with other potential contaminants of the soil. Indeed, it has been shown in the  
223 literature that addition of TiO<sub>2</sub>-NPs in sandy soils can improve Cu and Pb mobility in soils<sup>43,47</sup>. Sewage  
224 sludge also often contains a certain amount of heavy metals<sup>15</sup>; NP contamination of sewage sludge  
225 amended soils could thus represent a threat to both terrestrial and aquatic ecosystems through higher  
226 heavy metal bioavailability and leaching towards aquifers. Finally, different microbial communities  
227 likely occur in different soils, and might have a role on TiO<sub>2</sub> fate and speciation over long term  
228 exposure.

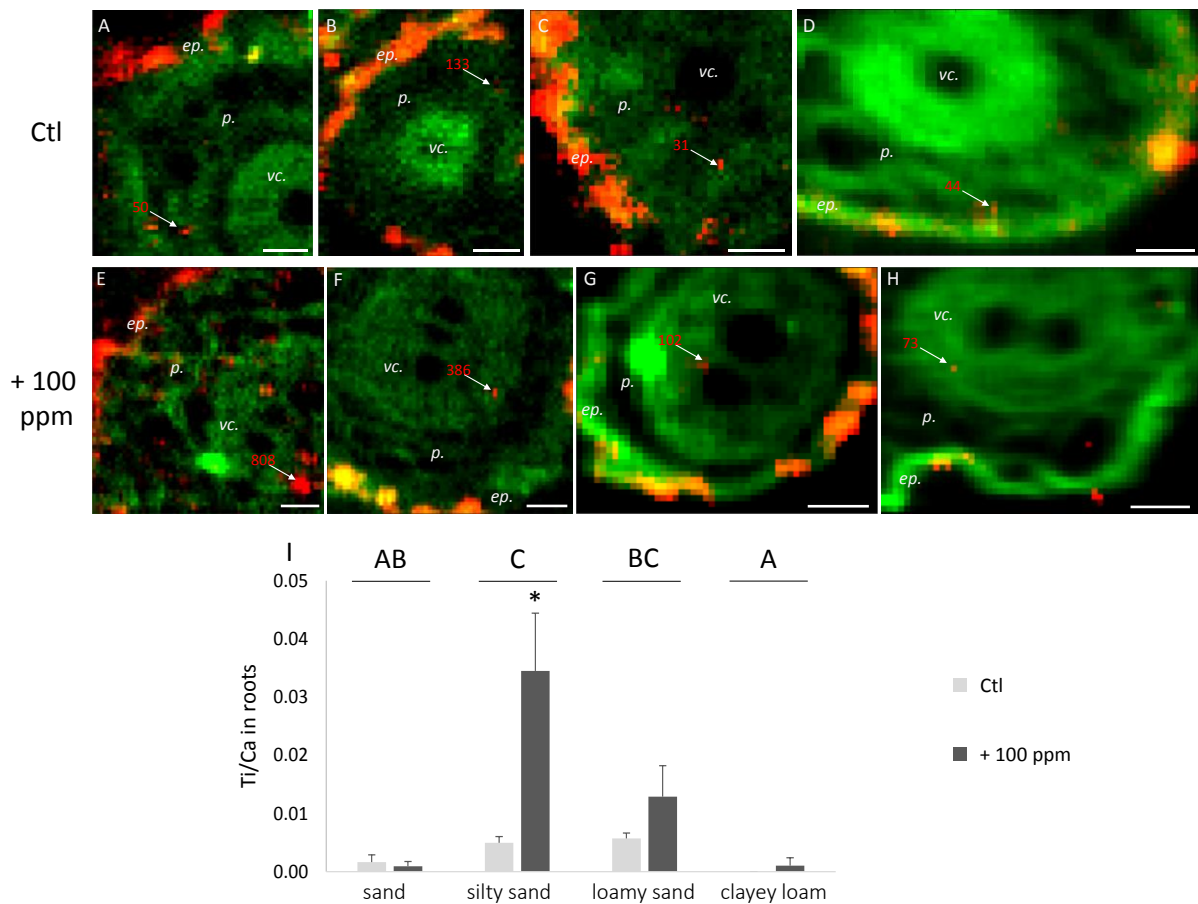
229

### 230 **3.2. Ti uptake in wheat roots**

231 Spatially resolved techniques were employed to investigate Ti uptake in wheat roots exposed in control  
232 soil and soil contaminated with 100 mg.kg<sup>-1</sup> of TiO<sub>2</sub>-NPs (the most environmentally relevant condition).

233 Figure 2A-D displays Ti distribution in roots of wheat seedlings grown in the 4 different natural soils. Ti  
234 was mainly detected adsorbed at the surface of the roots but also in the parenchyma with maximum  
235 fluorescence intensity of 42 counts in average for sand, loamy sand and clayey loam and up to 133  
236 counts in silty sand roots. In roots grown in contaminated soils (Figure 2E-H), the same distribution  
237 pattern appeared, with Ti mainly detectable on root surface and less Ti observed adsorbed in the clayey  
238 loam soil in comparison with the 3 other ones. However, the maximum fluorescence intensities were  
239 increased in root inner tissues (parenchyma and vascular cylinder) especially for roots grown in sand  
240 and silty sand. In first approach, these results seem to correlate with those obtained in the Ti  
241 partitioning experiment: Ti added to sandy soil is more available and thus internalized in roots, while  
242 Ti added in soils with more clay and organic matter is more retained by the soil matrix and lead only  
243 to a slight Ti fluorescence signal increase in wheat roots.

244 ICP-AES measurements of Ti content in roots would not be reliable because Ti can be heavily adsorbed  
245 on their surface, leading to over-estimation of Ti absorption. Thus, to have a proper quantitative  
246 assessment of Ti accumulation in roots, thin sections were analyzed using  $\mu$ PIXE coupled to  $\mu$ RBS,  
247 which permitted the quantification of all the chemical elements of the periodic table, light elements  
248 (C, H, O, N) included (Figure 2I). The main difference evidenced by the analysis was the one related to  
249 soil type ( $P = 0.0003$ ): roots from clayey soils contained significantly less Ti than the roots grown in  
250 loamy sand and silty sand. Wheat roots accumulated significantly more Ti after addition of  $\text{TiO}_2$ -NPs in  
251 silty sand only ( $P = 0.0008$ ). In this condition, Ti content was multiplied by a factor of 7. Surprisingly, in  
252 contrast with  $\mu$ XRF results, Ti content in roots grown on sand did not show any significant increase ( $P$   
253  $= 0.4699$ ). However, this is in agreement with the fact that Ti content in sand after addition of 100  
254  $\text{mg.kg}^{-1}$   $\text{TiO}_2$ -NPs did not increase significantly. One possible explanation for this discrepancy is that  
255  $\mu$ XRF technique has a lower limit of detection than  $\mu$ PIXE/RBS<sup>48</sup>. Overall, no correlation was detectable  
256 between Ti concentration in soil and Ti/Ca content in roots ( $r=0.111$ ,  $P = 0.742$ ).



257

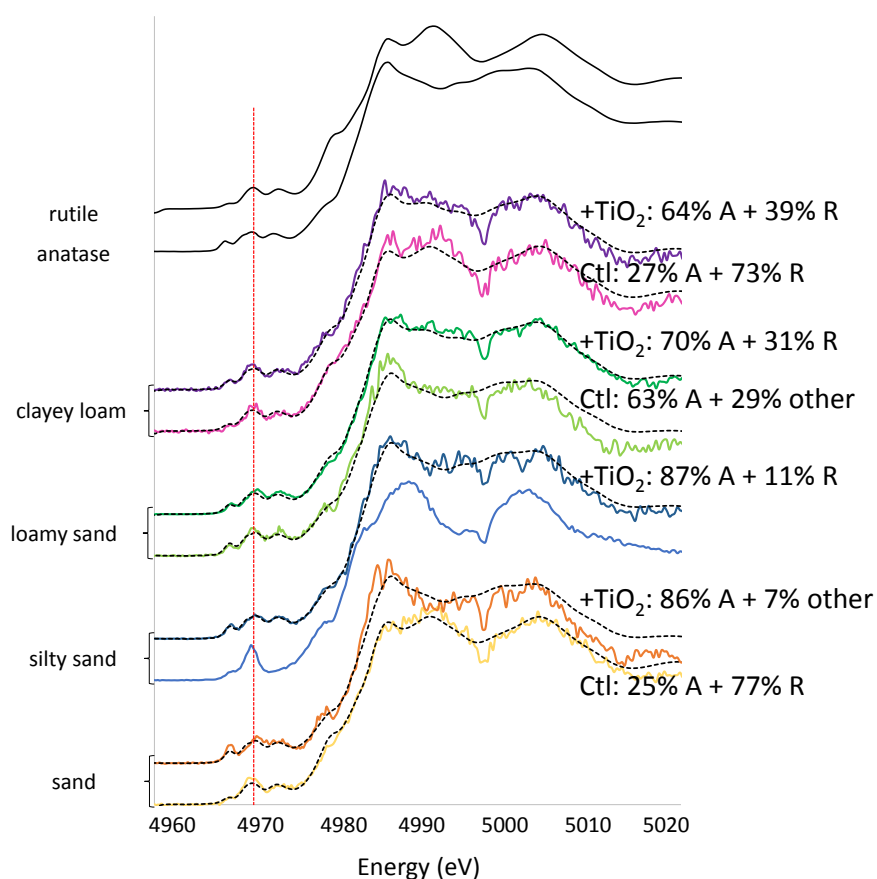
258 *Figure 2. Ti in wheat roots after a 3 week exposure: elemental distribution by  $\mu$ XRF of Ti (in red) and Ca*  
 259 *(in green) in root sections of wheat grown in control soils (A-D) or in contaminated soils (E-F). Numbers*  
 260 *in red (fluorescence counts) and arrows indicate the highest fluorescence intensity detected inside the*  
 261 *root. ep. epidermis, p. parenchyma, v.c. vascular cylinder. Scale bar = 50  $\mu$ m. Semi-quantitative analysis*  
 262 *of Ti by  $\mu$ PIXE/RBS in root sections of wheat grown in natural soils and soils contaminated with 100*  
 263 *mg.kg<sup>-1</sup> TiO<sub>2</sub>-NPs (I). Capital letters indicate significant differences among soil types and \* indicate*  
 264 *significant differences between exposed plants and their corresponding control (p<0.05) (n=3).*

265

266 Ti speciation was then analyzed by  $\mu$ XANES on high intensity spots identified by  $\mu$ XRF (Figure 3). This  
 267 analysis was performed in roots grown in the 4 different types of soils and in both natural and  
 268 contaminated (100 mg.kg<sup>-1</sup> added NPs) conditions. Different chemical forms of Ti were detected in  
 269 wheat grown in natural soils from mainly rutile in clayey loam and sand (with respectively 73 and 77%

270 of rutile crystalline form) to mainly anatase in loamy sand (63% anatase). Anatase and rutile forms of  
271  $\text{TiO}_2$  correspond to an octahedral arrangements of the O atoms around Ti. In the natural silty sand,  
272 there is a shift of the A3 peak towards the lower energies (about 0.5 eV) with an increase of the peak  
273 height and area which suggests a different conformation of the TiO unit going from octahedral towards  
274 trigonal bipyramidal (5 coordinations) or tetrahedral (4 coordinations) conformations <sup>49</sup>. After  
275 exposure to  $\text{TiO}_2$ -NPs (89% anatase, 11% rutile, Figure S1), the ratio between anatase and rutile in  
276 comparison with the same ratio in the control roots increased in the roots of all 4 conditions  
277 highlighting the contribution of the mainly anatase added NPs. The increase was more important in  
278 the case of plants grown in the soils with coarser texture (sand and silty sand).

279 There are not many studies evaluating NP fate in plants after soil exposure even though this number  
280 has been increasing over the last few years. But there are even fewer articles reporting the fate of NPs  
281 in roots (Majumdar *et al.* 2016 on  $\text{CeO}_2$ -NPs <sup>50</sup> or Gao *et al.*, 2018 on  $\text{CuO}$ -NPs<sup>51</sup>). Indeed it is very  
282 challenging to obtain this type of data and only spatially resolved techniques are able to provide  
283 reliable data getting rid of the geogenic signal surrounding the roots. Moreover, if one wants to  
284 perform a comparative study, the technique also needs to be quantitative which leaves the scientific  
285 community with few tools mainly based on spectroscopy. In this study by combining 4 different  
286 techniques namely  $\mu\text{XRF}$ ,  $\mu\text{PIXE/RBS}$  and  $\mu\text{XANES}$ , we were able to map Ti distribution in wheat roots  
287 with a good sensitivity, quantify it and investigate its speciation *in vivo*. Our results demonstrate that  
288 the added  $\text{TiO}_2$ -NPs can be taken up by plant roots when exposure occurs in soil and in particular in  
289 sand and silty sand. Considering the concentration of NPs added to natural soils (no significant increase  
290 of Ti concentration in soils after addition of  $100 \text{ mg}\cdot\text{kg}^{-1}$ , Figure 1A), our results also suggest that Ti  
291 from NPs was taken up by roots preferentially than geogenic Ti (no significant Ti concentration increase  
292 in soil vs. increase in roots). This highlights that the added Ti had a different behavior than the natural  
293 one and a different bioavailability which justifies the fact to study the impact of a  $\text{TiO}_2$ -NP  
294 contamination in the environment even though plants have evolved with high Ti concentrations in soils  
295 for ages.



296  
 297 *Figure 3. Ti speciation assessed by  $\mu$ XANES in wheat roots grown in control soils (Ctl) or soils*  
 298 *contaminated with  $100 \text{ mg.kg}^{-1} \text{ TiO}_2\text{-NPs (+TiO}_2\text{) after a 3 week exposure. A. anatase, R. rutile, other$*   
 299 *gathers Ti-acetate, Ti-oxysulfate and amorphous Ti oxide. In colors: experimental spectra, in dashed*  
 300 *black: fits. Red dashed vertical line shows the position of A3 peak in  $[\text{Ti}]_6$  spectra. The fit of Ctl silty sand*  
 301 *is not displayed since a major reference compound was not available (trigonal bipyramidal and/or*  
 302 *tetrahedral form(s)) for a proper fitting.*

303  
 304 Moreover, the results obtained in this work are also in agreement with several similar articles of the  
 305 literature. For instance, in a study carried out with the same NPs (but much higher concentration) and  
 306 a soil similar to our silty sand, Ti based NPs have been detected (TEM-EDX) in the roots of red clover  
 307 exposed to  $1000 \text{ mg.kg}^{-1}$  after 14 weeks of exposure<sup>52</sup>. Previously in 2011, Du *et al.*, also detected by  
 308 TEM,  $\text{TiO}_2\text{-NPs}$  in the roots of wheat grown for 2 weeks in an artificially contaminated loamy clay soil<sup>53</sup>.  
 309 Finally those results are also in line with those we previously demonstrated for wheat seedlings



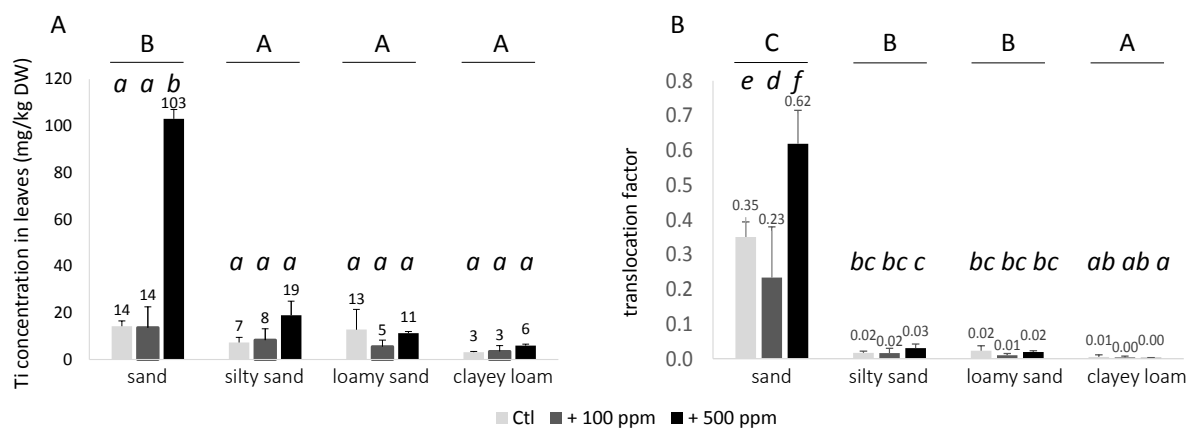
310 exposed for 7 days to the same nanomaterial in hydroponics: anatase TiO<sub>2</sub>-NPs were also evidenced in  
311 the roots by various techniques <sup>54,55</sup>.

312

### 313 **3.3. Ti translocation to wheat leaves**

314 Ti accumulation in aerial parts was quantified by ICP-AES (Figure 4A). Wheat leaves had a basal Ti  
315 concentration of 9 mg.kg<sup>-1</sup> in average in control soils. The only significant Ti concentration increase in  
316 leaves after exposure was detected for seedlings grown in sand after addition of 500 mg.kg<sup>-1</sup> TiO<sub>2</sub>-NPs  
317 ( $P < 0.0001$ ) with a concentration reaching more than 100 mg Ti.kg<sup>-1</sup> DW. The same trend was also  
318 noticeable in silty sand but not significant (from 7 in natural soil to 19 mg.kg<sup>-1</sup> in the most contaminated  
319 condition). Plants grown on clayey loam had the lowest Ti concentration in all conditions (4.4 mg.kg<sup>-1</sup>  
320 on average) even though this was the soil with the highest Ti concentration.

321 Another factor of interest after contamination of an agro-ecosystem is the translocation factor which  
322 corresponds to the fraction of Ti the plant is able to transfer from the soil to its leaves (Figure 4B). Even  
323 though the clayey loam soil was the richest in Ti – mainly geogenic Ti as demonstrated earlier – it was  
324 also the one in which Ti translocation factors were significantly lower than in other soils with an  
325 average of 0.4% ( $P < 0.0001$ ). The addition of TiO<sub>2</sub>-NPs to silty sand, loamy sand and clayey loam had  
326 no influence on Ti translocation factors whatever the concentration added. Translocation factor was  
327 only increased after addition of NPs in sand ( $P = 0.0108$ ). Sand was the soil with the lowest Ti content,  
328 but with the highest translocation factors ranging from 23.4 to 61.9% meaning that Ti was more  
329 bioavailable in sand soil. All conditions considered, a negative significant correlation was evidenced  
330 between Ti in soil and Ti in leaves ( $r = -0.374$ ,  $P = 0.0130$ ).



331  
 332 *Figure 4. A. Ti quantification in leaves and B. Translocation factors ( $[Ti]_{leaf}/[Ti]_{soil}$ ) determined by ICP-*  
 333 *AES in seedlings grown in control soils and soils contaminated with 100 or 500 mg.kg<sup>-1</sup> TiO<sub>2</sub>-NPs, after*  
 334 *a 3 week exposure. Lowercase letters indicate significant differences ( $p<0.05$ ) among NP*  
 335 *concentrations (control, 100 or 500 mg.kg<sup>-1</sup>) within a soil type in A or among all treatments in B. Capital*  
 336 *letters indicate significant differences among soil types. (n=6)*

337  
 338 Results in the literature also report very little to no Ti translocation to leaves when plants were exposed  
 339 in soils. For instance, tomato plants exposed to 250 mg.kg<sup>-1</sup> 25 nm TiO<sub>2</sub>-NPs in calcinated fuller earth  
 340 for 66 days accumulated between 25 to 83 mg Ti.kg<sup>-1</sup> approximately in their aerial parts with no  
 341 significant difference with the control condition<sup>56</sup>. Likewise, Gogos *et al.* did not evidenced any  
 342 significant increase in red clover shoot and wheat grain after exposure to the same TiO<sub>2</sub>-NPs as ours in  
 343 a silty sand contaminated with concentrations ranging from 1 to 1000 mg.kg<sup>-1</sup><sup>52</sup>. TiO<sub>2</sub>-NPs were also  
 344 not detected in the leaves of wheat plants grown on a contaminated loamy clay (clay: 32.6%, OM  
 345 content: 4.6%, added NPs: 90 mg.kg<sup>-1</sup>) for 2 weeks<sup>53</sup>. Ti was detected up to the fruits of cucumber  
 346 plants after exposure to TiO<sub>2</sub>-NPs in a sandy loam soil contaminated with 750 mg.kg<sup>-1</sup> for 150 days but  
 347 with no quantitative data or comparison to control<sup>57</sup>. Results were also similar for other NPs and plant  
 348 species (CuO and lettuce<sup>58</sup>, CeO<sub>2</sub> and radish<sup>59</sup>), unless very high concentrations were introduced (>1000  
 349 mg.kg<sup>-1</sup>).

350 Others in the literature also investigated soil characteristic influence on NP translocation, in particular  
351 the impact of organic matter. Interestingly most of the available studies concluded that increasing  
352 organic matter leads to higher metal concentration in plant shoots (Ce in kidney bean <sup>50</sup>, Ce in fescue  
353 <sup>60</sup>, Zn in cucumber <sup>61</sup>). However, in another study, about 10 times more Zn was found in the shoot of  
354 wheat grown on “acid soil” (with 1.6% OM and 8% clay) than in “calcareous alkaline soil” (with 4.1%  
355 OM and 28% clay) after exposure to ZnO NPs. This was related to a higher proportion of soluble Zn in  
356 the acidic soil (200 times more) <sup>62</sup>. Authors tried to confirm the relationship between OM and metal  
357 translocation by comparing Zn internalization in plants grown on sand with increasing concentrations  
358 of humic acid, but they were not able to reproduce the results obtained in natural soil: adding humic  
359 acid did not influenced Zn internalization. Finally, corn plants exposed to CeO<sub>2</sub> NPs in untreated soil  
360 contained significantly less Ce in roots but more Ce in shoot than plants exposed in the same soil  
361 enriched in organic matter. In parallel, ICP measurements demonstrated that the organic soil  
362 contained more Ce in the soil solution than the untreated soil (375 vs. 18 mg/100 mL soil solution) <sup>63</sup>  
363 which is in contradiction with the previous study. Thus it seems that the correlation between OM  
364 content and NP/metal uptake is still not clear. OM could also have an indirect effect on plant  
365 development, possibly leading to an increased foliar area and thus plant evapotranspiration which  
366 could in turn increase NP uptake by plants. Those discrepancies might also be related to plant species  
367 and their different strategies for metal uptake (monocot vs. dicot <sup>64</sup>) or related to NP surface charge  
368 or biotransformation. Indeed, XANES studies have demonstrated that Zn is mainly detected in soil and  
369 plant shoot as dissolved ions recomplexed to either phosphate, phytate, citrate or histidine <sup>65,66</sup> while  
370 Ce has been detected as Ce(IV)O<sub>2</sub>-NPs in roots and Ce(III) carboxyl in the stem and leaves, suggesting  
371 that the dissolution of CeO<sub>2</sub>-NPs took place in the plants rather than in the soil. Ce would then be  
372 present in the soil as NPs while Zn would be under ionic form which implies different interactions with  
373 the soil particles and thus different uptake dynamics. Ti XANES spectra in plant leaves, in this study  
374 and in our previous work, were dominated by the anatase NP crystalline form which indicates that

375 TiO<sub>2</sub>-NPs are more inert than ZnO-NPs and would behave in the soil more like CeO<sub>2</sub>-NPs. Obviously  
376 more studies are needed to really understand how OM impacts different types of NP uptake by plants.  
377 Soil texture, and in particular the amount of clay, is another parameter of interest to determine the  
378 fate of NPs. Our results showed that an increased percentage of clay in the soil lead to a decrease of  
379 Ti uptake in plant shoots. They are in agreement with a study from Zhang *et al.*, in which radish roots  
380 and shoots tend to accumulate more Ce after exposure to CeO<sub>2</sub>-NPs in the loamy sand (76% sand) than  
381 in the silty loam (16% sand) <sup>59</sup>. Likewise, Layet *et al.*, concluded that increasing clay fraction enhanced  
382 the retention of CeO<sub>2</sub>-NPs in soil and thus decreased its uptake by fescue <sup>60</sup>.

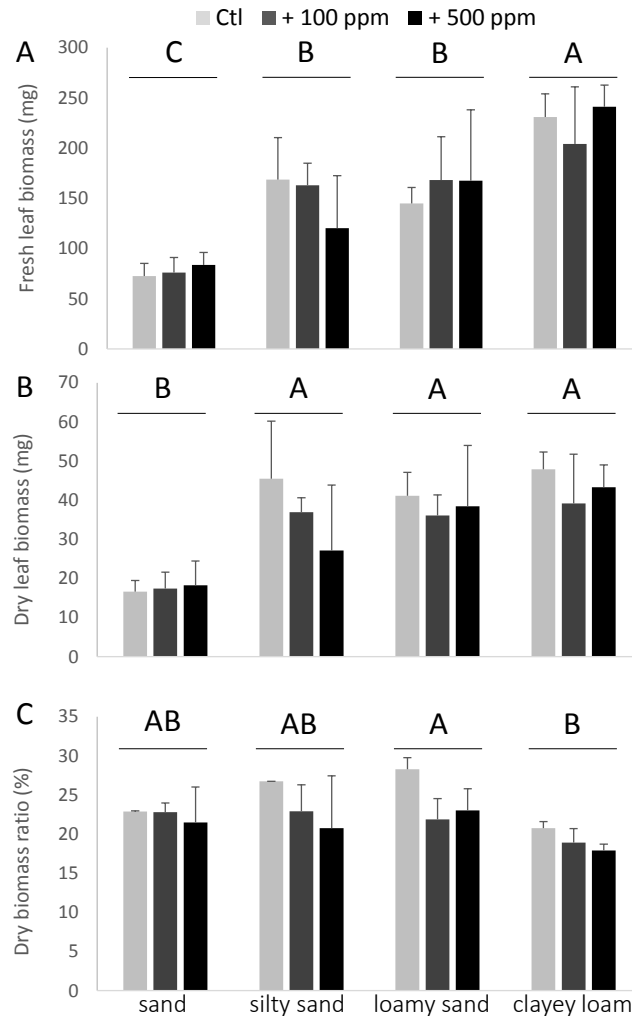
383

#### 384 **3.4. TiO<sub>2</sub>-NP phytotoxicity**

385 TiO<sub>2</sub>-NP toxicity was evaluated during the time course of the exposure via seedling shoot length and  
386 chlorophyll content. The main noticeable difference was linked to soil type with plants growing in sand  
387 being the smallest followed by plants grown in silty sand, loamy sand and clayey loam (for instance at  
388 day 16,  $P < 0.0001$ ) (Figure S3) but no significant difference was highlighted between NP treatment (for  
389 instance at day 16,  $P = 0.528$ ). Results for chlorophyll content were similar (data not shown): the only  
390 difference was related to soil type ( $P = 0.0080$ ) but not to NP addition ( $P = 0.9290$ ).

391 After 3 weeks, leaf fresh and dry biomasses were recorded (Figure 5). Fresh leaf biomass was only  
392 influenced by soil type ( $P < 0.0001$ ) with 3 groups of individuals: sand < silty sand and loamy sand <  
393 clayey loam (Figure 5A). Dry leaf biomass results were similar ( $P = 0.0008$ ) with plants grown in sand  
394 having the lowest biomass with 20 mg on average, followed by plants grown in silty sand, loamy sand  
395 and clayey loam with respectively 36 mg, 41 mg and 43 mg (Figure 5B). The ratio between fresh matter  
396 and dry matter seems to be a more sensitive parameter which evidenced a soil effect ( $P = 0.0080$ ):  
397 plants grown on clayey loam had 19% of dry matter vs. plants grown in loamy sand with 25% (Figure  
398 5C). Moreover, this ratio also highlighted a NP concentration effect ( $P = 0.0410$ ) with plants exposed in

399 soil with 500 mg.kg<sup>-1</sup> TiO<sub>2</sub>-NPs having a lower content in dry matter as compared with plants grown in  
 400 control soils. It has already been shown previously that NPs are able to interact with water uptake  
 401 processes <sup>67</sup>.



402  
 403 *Figure 5. TiO<sub>2</sub>-NP phytotoxicity on wheat biomass (fresh: A, dry: B, ratio between fresh and dry: C) after*  
 404 *a three week exposure to different concentrations of NPs (0, 100, 500 mg.kg<sup>-1</sup>) in different types of soils.*  
 405 *Capital letters indicate significant differences (p<0.05) among soil types (n=6).*

406  
 407 TiO<sub>2</sub>-NP impact on plant hydromineral nutrition was also investigated at harvest after ICP-AES  
 408 measurements of plant macro- (Ca, K, P, S) and micro- (Al, B, Ba, Cu, Fe, Mg, Mn, Na, Si, Zn) nutrient  
 409 concentrations. Again, the main effect detected was a soil effect (Manova with Pillai's trace, F<sub>3,32</sub>= 10.4,  
 410 P < 0.0001) but no NP specific effect was highlighted (Manova with Pillai's trace, F<sub>2,33</sub>= 1.5, P = 0.1210).

411 In the literature, no major acute toxicity has been reported after plant exposure to TiO<sub>2</sub>-NPs in soil. For  
412 instance, there was no significant difference in catalase and ascorbate peroxidase activities in  
413 cucumber exposed to up to 750 mg.kg<sup>-1</sup> TiO<sub>2</sub>-NPs. Likewise, no effect on chlorophyll content was  
414 detected until 500 mg.kg<sup>-1</sup> NPs (but an increase was seen at 750 mg.kg<sup>-1</sup>)<sup>57</sup>. The same results were also  
415 obtained previously in hydroponics for short term exposure<sup>29,54,55</sup>. It would be interesting to confirm  
416 this lack of toxicity through multigenerational tests and through more in-depth endpoints (-omics  
417 approaches).

418

### 419 **Conclusion**

420 The results obtained in this study demonstrated that TiO<sub>2</sub>-NPs can be significantly taken up by wheat  
421 seedlings after a 3 week exposure in a sandy soil in which 500 mg.kg<sup>-1</sup> of engineered NPs have been  
422 added. This is also in this soil type that NPs were able to reach in higher quantities the leachates. Thus  
423 if a contamination occurs in a sand, NPs will be very mobile (towards aquifers) and bioavailable to crop  
424 plants representing a risk for trophic transfer. However, sand is not a relevant medium for agriculture.  
425 The same trend has been highlighted in silty sand but in smaller proportions. In contrast, in loamy sand  
426 and clayey loam no difference has been evidenced after addition of NPs to the system: no Ti  
427 concentration increase in the soil, partly due to the high Ti geogenic background, no increase in plant  
428 roots and leaves and little to no increase in the soil leachates. This difference in TiO<sub>2</sub>-NP behavior can  
429 be related to 3 characteristics of the soil used: organic carbon content, clay content and CEC, but the  
430 experimental plan do not allow to go further. In all conditions, no acute sign of phytotoxicity was  
431 detected in the endpoints analyzed. The overall conclusion for agro-ecosystems is that TiO<sub>2</sub>-NP  
432 contamination (after a short term exposure: 3 weeks) seems to have little impact on plant health and  
433 low chance of uptake as well as low risk of leaching to the aquifers. The soil displaying the highest risk  
434 for food safety would be the silty sand but this soil has little nutrients for plant growth and is not the  
435 best featured for agriculture. However, those results also imply that TiO<sub>2</sub>-NPs will remain in the soil  
436 with possible implications for soil micro and macro-organisms. It is thus important to understand NP

437 fate in soils upon weathering on a long term experiment. Finally, an experiment covering the full life  
438 cycle of different types of crop plants (fruit crop, leaf crop, root crop) is also needed to confirm data  
439 obtained here and further investigate the potential risk on silty sand soil.

440

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444

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450

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452

453 Supporting Information is available, it contains 4 figures and 2 tables.

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