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## Assessing the impacts of sewage sludge amendment containing nano-TiO<sub>2</sub> on tomato plants: A life cycle study

Mansi Bakshi, Clarisse Liné, Diana Bedolla, Ricardo José Stein, Ralf Kaegi, Géraldine Sarret, Ana Pradas del Real, Hiram Castillo-Michel, P.C. Abhilash, Camille Larue

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1 **Assessing the impacts of nano-TiO<sub>2</sub> on tomato plants in a sewage sludge amended soil: A**  
2 **life cycle study**

3  
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6

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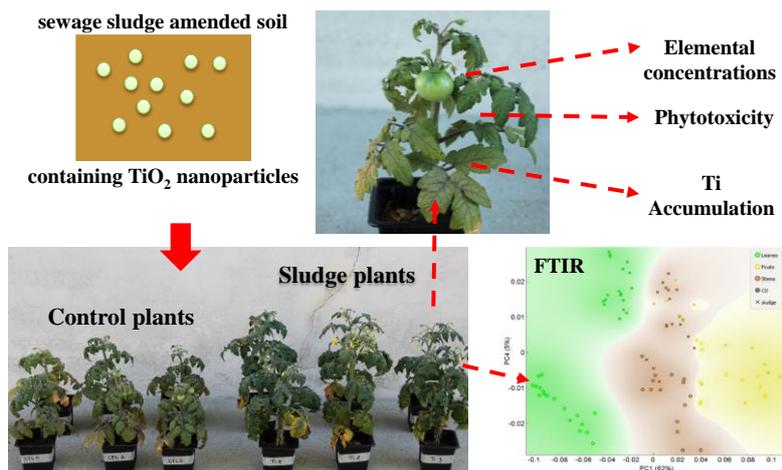
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26 **Graphical abstract:**



27

28 **Abstract**

29 Increasing evidence indicates the presence of engineered nanoparticles (ENPs) in sewage  
30 sludge derived from wastewater treatment. Land application of sewage sludge is, therefore,  
31 considered as an important pathway for ENP transfer to the environment. The aim of this  
32 work was to understand the effects of sewage sludge containing nano-TiO<sub>2</sub> on plants (tomato)  
33 when used as an amendment in agricultural soil. We assessed developmental parameters for  
34 the entire plant life cycle along with metabolic and bio-macromolecules changes and titanium  
35 accumulation in plants. The results suggest that the sewage sludge amendment containing  
36 nano-TiO<sub>2</sub> increased plant growth (142% leaf biomass, 102 % fruit yield), without causing  
37 changes in biochemical responses, except for a 43% decrease in leaf tannin concentration.  
38 Changes in elemental concentrations (mainly Fe, B, P, Na, and Mn) of plant stem, leaves and,  
39 to a lesser extent fruits were observed. Fourier-transformed infrared analysis showed  
40 maximum changes in plant leaves (decrease in tannins and lignins and increase in  
41 carbohydrates) but no change in fruits. No significant Ti enrichment was detected in tomato  
42 fruits. In conclusion, we evidenced no acute toxicity to plants and no major implication for  
43 food safety after one plant life cycle exposure.

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52 **1. Introduction**

53 Engineered nanoparticles (ENPs, particle having at least one external dimension between 1-  
54 100 nm) have many industrial applications ranging from food packaging to remediation of  
55 groundwater, wastewater and soil<sup>1,2</sup>. Among all ENPs, nano-TiO<sub>2</sub> are one of the most  
56 produced alongside with Ag and ZnO<sup>3</sup>. Their photocatalytic activity, high stability and  
57 anticorrosion properties make them suitable for a wide range of applications<sup>4-6</sup>. Nano-TiO<sub>2</sub>  
58 can be used in cosmetics and skin care products like sun blocks, toothpastes, food,  
59 pharmaceuticals, paints, water treatment processes and cleaning air products<sup>7-10</sup>. As bulk  
60 material (possibly containing a fraction of ENPs), TiO<sub>2</sub> is widely used as a pigment<sup>7</sup>. With a  
61 constant rise in the production of ENPs, they will unavoidably make their way to the  
62 environment. Agricultural soil, in particular, is a very sensitive compartment and a main sink  
63 for ENPs<sup>3</sup>. Two major pathways are responsible for ENP input into agricultural soil: firstly,  
64 ENPs contained in sewage sludge applied as fertilizer in agriculture results<sup>11,12</sup>. Indeed, many  
65 studies have already evidenced ENP presence in wastewater treatment plants<sup>13,14</sup> and further  
66 in biosolids<sup>10</sup>. Secondly, the use of ENPs in plant protection products (nanoencapsulated  
67 pesticides, nanoformulations and nano-based carrier systems) leads to their intentional input  
68 in soil<sup>15-18</sup>. A probabilistic material flow analysis predicted nano-TiO<sub>2</sub>, among other ENPs, to  
69 be present in the highest concentrations in the environment<sup>3</sup>. In particular, the modelled  
70 concentration of nano-TiO<sub>2</sub> in sewage sludge is predicted to be 1.60 g/kg while for sludge  
71 amended soil the concentration is estimated up to 61 mg Ti/kg. This study indicates that the  
72 concentrations of nano-TiO<sub>2</sub> in biosolids and effluent treated soils have increased 30–40 times  
73 between 2005 and 2012<sup>3</sup>, highlighting the importance of studying the fate of nano-TiO<sub>2</sub> in  
74 sewage sludge fertilized soil.

75 Sewage sludge is widely used in agricultural fields as an alternative to chemical fertilizers  
76 due to its high nutrient quality. This use of sewage sludge represents a highly sustainable  
77 strategy, since it addresses two important global challenges: efficient agriculture  
78 production thanks to inputs in micro and macronutrients and organic matter, as well as  
79 disposal of large quantities of waste. However, this entails the risk of toxicity and food chain  
80 contamination due to the presence of heavy metals and organic pollutants in the sewage  
81 sludge<sup>19</sup>. The European Union (Directive 86/278/EEC) and the U.S.A. (Title 40 Code of Federal  
82 Regulations, Part 503) have legislations defining permissible limits for heavy metal in sewage  
83 sludge used for land application<sup>20,21</sup>. While ENPs are emerging as possible pollutant with  
84 unknown risks, no regulation exists so far for governing their release in the environment<sup>22-</sup>  
85 <sup>24</sup>.

86 Despite the risk of food chain transfer and biomagnification through uptake, translocation  
87 and accumulation of ENPs in plants and the wide use of sewage sludge as amendments, there  
88 is limited research on the impact of ENPs on plants through application of sewage sludge  
89 amended soil. Recently, several studies have investigated the phytotoxicity of pristine nano-  
90 TiO<sub>2</sub> on plants<sup>9,25-29</sup>. Du et al. (2011)<sup>30</sup> observed that nano-TiO<sub>2</sub> decreased plant biomass in  
91 wheat and penetrated cell walls, plant epidermis via apoplastic pathway and inhibited soil  
92 enzyme activity. Servin et al. (2013)<sup>31</sup> reported root-to-fruit translocation of nano-TiO<sub>2</sub> in  
93 cucumber grown on a spiked soil. More recently, we observed no acute phytotoxicity in terms  
94 of growth, biomass and chlorophyll concentration on wheat plants grown in nano-TiO<sub>2</sub> spiked  
95 soil, but a significant Ti uptake in sandy soils<sup>32</sup>. While some studies have explored the toxicity  
96 of nano-TiO<sub>2</sub> on soil related organisms, studies investigating the impacts of nano-TiO<sub>2</sub>  
97 resulting from sewage sludge applications on sewage sludge microbial communities, soil  
98 macro-organisms and plants are very few<sup>33-35</sup>. Thus, the predicted increase in nano-TiO<sub>2</sub>

99 production volumes<sup>36</sup> warrants further investigations on fate of nano-TiO<sub>2</sub> in sewage sludge  
100 and associated implications for food safety.

101

102 The objective of the present study was to develop a realistic exposure scenario simulating the  
103 growth of edible plants on agricultural soil treated with sewage sludge containing nano-TiO<sub>2</sub>.  
104 Sewage sludge was produced in a pilot wastewater treatment plant treating municipal  
105 wastewater at the Swiss Federal Institute of Aquatic Science and Technology (EAWAG,  
106 Dubendorf, Switzerland). Monitoring the entire life cycle of tomato plants grown on this  
107 sewage sludge amended soil, we investigated the impact on (i) plant growth and development  
108 (ii) biochemical responses (iii) changes in biomacromolecules (lipids, proteins, carbohydrates)  
109 using Fourier Transformed Infra-Red spectroscopy (FTIR) (iv) effect on plant elemental  
110 concentrations and (v) Ti accumulation by inductively coupled plasma-optical emission  
111 spectrometry (ICP-OES).

112

## 113 **2. Materials and Methods**

### 114 **2.1. Soil and sewage sludge description**

115 The sewage sludge was produced in a pilot wastewater treatment plant at EAWAG<sup>37</sup>. This  
116 sludge comes from the Dubendorf city, a suburban town of Zurich in Switzerland. During a  
117 previous experiment, nano X-ray fluorescence (XRF) performed on ID16 beamline at the  
118 European synchrotron radiation facility (ESRF) evidenced the presence of micro- and nano-  
119 TiO<sub>2</sub> in the sewage sludge (26% ENPs)<sup>38</sup>. X ray absorption spectroscopy (XANES) performed  
120 on ID21 (ESRF) showed that Ti was present in the sludge as 55 ± 1% anatase and 45 ± 2% rutile.  
121 The top layer (0–30 cm) of a loamy soil (38% sand, 42% silt and 20 % clay) was collected at La  
122 Côte Saint-André (Isère, France) from a field under permanent pasture to serve as “control

123 soil". Control soil contained  $37 \pm 2\%$  anatase,  $49 \pm 7\%$  rutile and  $14 \pm 6\%$  amorphous  $\text{TiO}_2$ <sup>38</sup>.  
124 Both sludge and soil were previously characterized<sup>39</sup> and the main physico-chemical  
125 properties are given in supplementary information (SI). A mixture of soil and sewage sludge  
126 (w/w) was prepared in the ratio of 1:10 (sludge: soil). This was considered as "sludge amended  
127 soil". In this condition, 20% of the Ti was in nanoparticulate form (<100 nm) and about 50%  
128 in the 100-300 nm range<sup>38</sup>.

129

## 130 **2.2. Plant exposure**

131 Twelve experimental units were filled with 200 g of sludge amended soil or control soil (six  
132 biological replicates per condition). Seeds of tomato plants (*Solanum lycopersicum*, var. Red  
133 Robin, provider: Germinance) were sown and grown until the ripening of tomato fruits with  
134 watering *ad libitum*. This tomato variety was selected because of its small size (dwarf variety)  
135 and short life cycle. All experiments were carried out in a growth chamber under controlled  
136 conditions: day/night photoperiod (16/8 h),  $100 \mu\text{mol}/\text{m}^2/\text{s}$ , day/night temperature ( $24/20 \pm 1$   
137 °C), and day/night relative humidity (70/75%). The development parameters (number of  
138 leaves, flowers, fruits, plant height) were monitored throughout the whole plant life cycle.  
139 Chlorophyll level was assessed everyday (SPAD-502 chlorophyll meter, Minolta Camera Co.,  
140 Japan). After 4 months (120 days), plants were harvested, fresh biomass was recorded and  
141 plants were split in three. Fresh leaves for biochemical analyses were weighed, immediately  
142 frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until analysis. The remaining leaves along with  
143 stems and fruits were dried and ground for determination of elemental concentration by ICP-  
144 OES. Finally, for the assessment of biomacromolecular changes by FTIR the dried samples  
145 were further grinded using a Fast Prep® grinder.

146

147 **2.3. Analysis of biochemical responses**

148 Tomato leaves were analysed for six different biomarkers. Three biochemical responses were  
149 investigated which were oxidative stress (lipid peroxidation), photosynthesis (chlorophyll a,  
150 b) and secondary metabolites (phenolic compounds, tannins and flavonoids). Lipid  
151 peroxidation was assessed according to Hodges et al. (1999)<sup>40</sup>. Chlorophyll a and b  
152 concentrations were determined by the method given by Lichtenthaler (1987)<sup>41</sup>. Phenolic  
153 compounds were estimated following Ainsworth and Gillespie (2007)<sup>42</sup>. Flavonoids were  
154 determined according to Settharaksa et al. (2014)<sup>43</sup> and tannins following El Euch et al.  
155 (2015)<sup>44</sup>. Detailed methods are described in SI.

156

157 **2.4. FTIR analysis**

158 FTIR was used to probe molecular vibrations in plant samples and thus gain information on  
159 the biochemical composition (lipids, carbohydrates, proteins). Dried plant stems, leaves and  
160 fruits were analysed on ID21 beamline at ESRF. Each powdered sample was analysed in ATR-  
161 mode using a diamond crystal (Thermo Nicolet, Nexus, Smart Orbit). Three independent  
162 technical replicates from each sample (6 biological replicates per treatment) were acquired.  
163 One spectrum is an average of 64 scans per sample, over a range of 4000 to 400  $\text{cm}^{-1}$  and  
164 with a spectral resolution of 4  $\text{cm}^{-1}$ . The full experimental setup for FTIR acquisition is given  
165 in SI. OMNIC software was used to export experimental spectra.

166 FTIR data treatment was performed using Orange software<sup>45</sup>. Briefly, data were pre-  
167 processed which implies selection of the region of interest (here 1755 – 455  $\text{cm}^{-1}$  range which  
168 contained most of the variability by looking at the average and standard deviation of the  
169 absorbance), vector normalization and smoothing by Savitzky-Golay filter. Principal

170 component analyses (PCA) were performed to visualize the data in a new coordinate space  
171 optimized for detection of differences between groups. Second derivative was performed to  
172 highlight spectral differences detected using PCA.

173

## 174 **2.5. Elemental concentration in soil, sewage sludge and plants**

175 ICP-OES was used to measure Ti and other micro and macro-elements in plant leaves, stems  
176 and fruits as well as in soils according to Larue et al. (2018)<sup>32</sup>. Blank (only chemicals) and  
177 standard reference material (NIST 1573a, tomato leaves) were used as controls in the  
178 digestion process.

179 Soil exchangeable and extractable Ti concentrations were determined through BaCl<sub>2</sub> and HCl  
180 extractions, respectively. Briefly, for soil exchangeable elements, 3 g of soil were mixed with  
181 10 mL of unbuffered 0.01 M BaCl<sub>2</sub>, shaken horizontally at 150 rpm overnight at room  
182 temperature. For extractable elements, 1 g of dry soil was extracted with 10 ml of 0.1M HCl,  
183 shaken horizontally at 150 rpm for 30 min at room temperature. Soil mixtures were then  
184 filtered through Whatman filter paper, dried and digested before analysis. Analyses were  
185 performed on an ICP-OES Spectro Arcos (Ametek).

186

## 187 **2.6. Statistical analysis**

188 Data reported were averages of six biological replicates  $\pm$  standard deviations (SD). Data were  
189 checked for normality (Shapiro's test) and homoscedacity (Bartlett's test). When assumptions  
190 were met for parametric analyses, a Student *t*-test was used. Otherwise, a Wilcoxon test was  
191 applied. In all cases the statistical significance was based on a p value < 0.05. Additionally, ICP-  
192 OES results were analysed using PCA to identify different profiles. All statistical analyses were  
193 performed using the R statistical software (version 3.1.3)<sup>46</sup> and vegan package<sup>47</sup> for PCA.

194

### 195 **3. Results and Discussion**

#### 196 **3.1. Titanium concentration in soil**

197 Titanium concentration for the sludge amended soil was  $819 \pm 62$  mg/kg and for control soil  
198  $801 \pm 102$  mg/kg (Figure S1 for other element concentrations). The exchangeable Ti  
199 concentration for control soil and sludge amended soil was 0.004% and 0.006%, respectively.  
200 The extractable soil fraction for both soils was 0.08%. No significant difference was found  
201 between the control and sludge amended soil for the total, extractable and exchangeable Ti  
202 ( $p > 0.05$ ).

203 Despite the fact that the control soil and sludge amended soil had similar Ti concentrations,  
204 Ti origin was different for both. While the control soil had geogenic Ti concentration from  
205 natural soil, the sludge amended soil had 9/10 natural Ti and 1/10 coming from the sludge  
206 which was most probably engineered  $\text{TiO}_2$ . Those two Ti forms might have different behaviour  
207 in the environment and different toxicity potentials. Indeed, natural  $\text{TiO}_2$  has a rough surface  
208 and is included in organo-mineral soil aggregates (with Si, Al, Fe), whereas  $\text{TiO}_2$  observed in  
209 the sludge is composed of homo and heteroaggregates dominated by organic matter<sup>38</sup>.

210 Properties such as organic matter content and soil texture (clay content) have been cited as  
211 important parameters controlling ENP behaviour in soil, influencing their mobility<sup>48</sup>. The  
212 presence of high organic matter content in the soil (8.8%) and in the sludge amended soil  
213 (10.9%) thus is a probable explanation for low fractions of both exchangeable and extractable  
214 Ti in the two media. Also, the agricultural soil was rich in clay fraction (20%) which further  
215 elucidates our results. Indeed, in our recent work investigating  $\text{TiO}_2$  ENP fate in 4 different  
216 types of soil (from sandy to clayey); clay content and organic carbon were found to influence

217 the transfer of Ti, with the soil with highest clay content (42%) displaying no ENP mobility  
218 compared to sand and silty sand soils<sup>32</sup>. Likewise, Gogos et al. (2016)<sup>49</sup> reported that leached  
219 Ti amount in a brown earth soil (sandy loamy to loamy fine fraction) was found to be  $10^{-4}$  %  
220 of the initial spiked Ti amount even at the highest concentration of 1000 mg/kg.

221

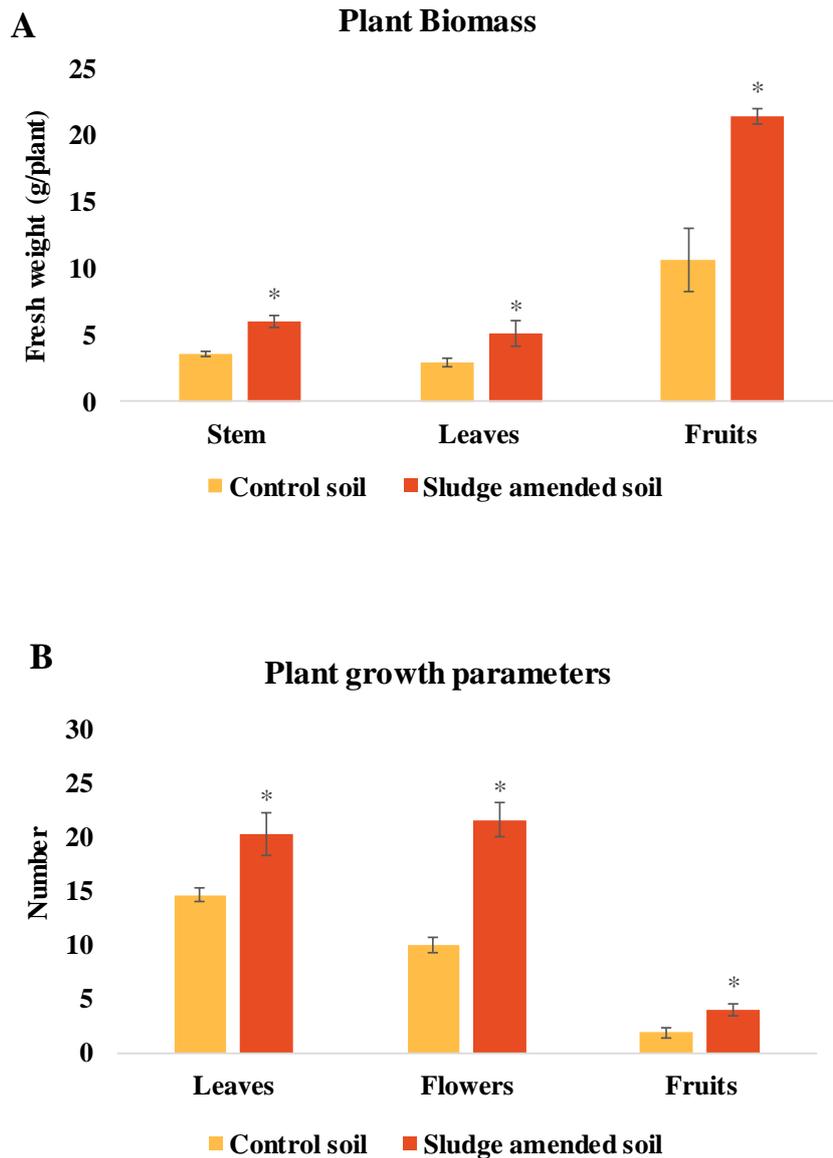
### 222 **3.2. Plant growth and developmental parameters:**

223 Plants grown in sewage sludge amended soil significantly increased biomass production for  
224 all organs (stem: +92 %, leaves: + 142% and fruits: + 102 %,  $p < 0.050$ ) as well as the number of  
225 leaves (20 per plant for sludge amended soil vs. 15 for control soil: +33%,  $p = 0.047$ ) and flowers  
226 (23 per plant in amended soil vs. 10 for control soil: +130%,  $p = 0.012$ ) compared to plants  
227 grown in the control soil (Figure 1A, B). Fruit yield was also significantly increased with an  
228 average of 4.4 fruits per plant amounting for 21.50 g in the sludge amended soil and 2.2 fruits  
229 per plant for a total weight of 10.63 g in the control soil ( $p = 0.010$  for fruit number and  $p = 0.005$   
230 for fruit biomass). The average plant height for sludge amended soil was  $20.68 \pm 1.42$  cm  
231 which was 43% higher than plants grown in the control soil ( $14.46 \pm 0.45$  cm,  $p = 0.012$ , data  
232 not shown). The monitoring of developmental parameters through the entire plant life cycle  
233 showed that after an average of 50 days of experiment, plants in the amended soil started  
234 exhibiting greater development compared to the control plants (Figure S2A, B, C).

235 In this study, we found that sewage sludge amendment containing nano-TiO<sub>2</sub> stimulated plant  
236 growth and tomato yield. Sewage sludge was rich in organic matter and contained substantial  
237 amount of nutrients. Total carbon, nitrogen, phosphorus and sulphur concentrations in  
238 sludge amended soil were multiplied by 3.4, 6.8, 1.8 and 23.6, respectively in comparison with  
239 control soil, thereby contributing to the fertilizer effect. The fact that sewage sludge

240 application is an effective mean for improvement of plant vegetative growth and soil  
241 properties has been widely reported <sup>50–52</sup>.

242 Some studies in the literature have explored the phytotoxicity of nano-TiO<sub>2</sub> using  
243 developmental parameters with contrasting results. nano-TiO<sub>2</sub> were found to induce longer  
244 roots in cucumber plants at a concentration of 750 mg/L<sup>31</sup> as well as increased germination  
245 rates in *Arabidopsis thaliana* seedlings (500 mg/L)<sup>53</sup>. However, Song et al. (2013a)<sup>54</sup> found no  
246 effects of nano-TiO<sub>2</sub> treatment on oilseed rape, lettuce and kidney bean at concentration  
247 range of 100 to 5000 mg/L. More recently, we evidenced no impact on wheat seedlings grown  
248 on different types of soils after exposure to 500 mg/kg<sup>32</sup>. Finally, in a sewage sludge study,  
249 Josko and Oleszczuk (2013)<sup>19</sup> reported that inclusion of 10 mg/kg TiO<sub>2</sub> ENPs in sewage sludge  
250 amended sandy soil inhibited root growth in plants *Lepidium sativum* and *Sinapis alba*.



251

252 **Figure 1: (A) Plant fresh biomass for stem, leaves and fruits and (B) number of leaves, flowers**

253 **and fruits for tomato plants grown for 120 days on control soil and sludge amended soil.**

254 **Asterisks represent significant difference ( $p < 0.05$ ) as indicated by Student T test ( $n = 6$ ).**

255

256 Still it is unclear how  $\text{TiO}_2$  ENPs affect plants; it seems that in most studies there was no clear

257 dose-response effects and that those effects are modulated by the soil properties. However,

258 bulk Ti has been considered as a biostimulant for a long time and has been used in

259 commercial foliar fertilizers for improving plant growth and development<sup>55</sup>. For instance, use

260 of Tytanit<sup>®</sup>, a Ti based foliar fertilizer, significantly improved the fruit yield of tomato at 960  
261 g Ti /ha<sup>56</sup>. In our study we concluded that the overall improved yield of tomato plants after  
262 sewage sludge amendment was attributed to the nutritive value of sludge, while we cannot  
263 be certain on the positive or negative effects of nano-TiO<sub>2</sub>.

264

### 265 **3.3. Biochemical responses**

266 No significant difference was observed in leaves for all the biomarkers related with secondary  
267 metabolites except tannins (Table 1). Leaf tannins significantly decreased from 3.09 ± 0.95 in  
268 control to 1.77 ± 0.13 mg CE/g f.wt in plants exposed to sewage sludge (p=0.022). Flavonoid  
269 concentration in control soil was 13.24 ± 5.04 and in sludge amended soil 12.02 ± 0.23 mg  
270 CE/g f.wt. The concentration for phenolic compounds for control soil and sludge amended  
271 soil was 10.54 ± 4.48 and 10.79 ± 3.56 µg GAE/g f.wt. respectively.

272 Among secondary metabolites, tannins, flavonoids and phenolic compounds are widely  
273 distributed in plants, and play a prominent role in general defence strategies (against  
274 herbivores, microbes, viruses, or competing plants), as well as contributing to food quality<sup>57</sup>.

275 Usually, a high tannin concentration favours plant growth in poor soil, in which the same plant  
276 species with a low tannin concentration could not grow<sup>58,59</sup>. A decrease in secondary  
277 metabolites might weaken the defence system of the plants and be a threat to plant survival  
278 in a more competitive environment (vs. in growth chamber). So far a very limited number of  
279 studies have investigated the impact of ENPs in general, and nano-TiO<sub>2</sub> in particular, on  
280 secondary metabolites.

281

282 ***Table 1: Values for flavonoids, phenolic compounds, tannins, chlorophylls a, b and***  
283 ***malondialdehyde (MDA) in leaves of tomato plants grown for 120 days on control and sludge***

284 **amended soils. Asterisks represent significant difference ( $p < 0.05$ ) as indicated by Student T**  
 285 **test ( $n=6$ ).** CE: catechin equivalent, GAE: gallic acid equivalent

286

<b>Biomarker</b>	<b>Control soil</b>	<b>Sludge amended soil</b>
Flavonoids (mg CE/g f.wt.)	13.24 ± 5.04	12.02 ± 0.23
Phenolic compounds (ug GAE/g f.wt.)	10.54 ± 4.48	10.79 ± 3.56
Tannins (mg CE/g f.wt.)	3.09 ± 0.95	1.77 ± 0.13*
Chlorophyll a (mg/g f.wt.)	1.37 ± 0.31	1.62 ± 0.62
Chlorophyll b (mg/g f.wt.)	0.89 ± 0.20	1.20 ± 0.40
MDA (nmol/g f.wt.)	3.84 ± 2.43	4.86 ± 1.64

287

288

289 For photosynthesis assessment, SPAD chlorophyll meter measurements recorded along plant  
 290 growth revealed no major difference in chlorophyll content (data not shown). This result was  
 291 further confirmed by assessing photosynthetic pigments in plant leaves at the end of plant  
 292 exposure: the average concentration of chlorophyll a and b was  $1.53 \pm 0.52$  ( $p=0.520$ ) and  
 293  $0.97 \pm 0.34$  mg/g f.wt. ( $p=0.623$ ) respectively. Previous studies have also reported similar  
 294 results where chlorophyll content in wheat<sup>60</sup> and tomato<sup>61</sup> was not impacted by TiO<sub>2</sub> ENP  
 295 treatment.

296 Finally, oxidative stress was assessed through lipid peroxidation: leaf malondialdehyde (MDA)  
 297 concentration in leaves did not change significantly ( $p=0.572$ ) with values from  $3.84 \pm 2.43$  in  
 298 control plants to  $4.86 \pm 1.64$  nmol/g f.wt in plants grown on sludge amended soil. Likewise,  
 299 Koce et al. (2014)<sup>62</sup> found no significant effect on anti-oxidative enzymes (glutathione

300 reductase, ascorbate peroxidase, guaiacol peroxidase, catalase) and lipid peroxidation levels  
301 in *Allium cepa* in a 24 hr study using a wide range of nano-TiO<sub>2</sub> concentration (0.1-1000 mg/L).  
302 MDA is the ultimate product of lipid peroxidation damage caused by generation of free  
303 radicals. Flavonoids and tannins are reported to exert inhibition of lipid peroxidation and  
304 scavenging of oxygen radicals<sup>58</sup>. Therefore, a decrease in tannin and flavonoid concentrations  
305 and a subsequent increase in MDA content might be indicative of a correlation between  
306 decreasing scavenging activity and increasing reactive oxygen species (ROS) concentration.  
307 Tomato plants might thus have gone through a moderate oxidative stress during plant growth  
308 as suggested by the significant decreased in tannin concentration and trend in increased MDA  
309 content (however, not significant upon harvest). The lack of biochemical response might also  
310 be associated with the duration of exposure. Indeed, a longer contact duration has been  
311 found to favour interactions between ENPs and sewage sludge components (*e.g.* organic  
312 matter) thereby reducing their bioavailability (and toxicity) through adsorption or  
313 aggregation<sup>19,63</sup>. Also it is possible that initially plants might have experienced stress but later  
314 adapted to it such that no significant difference was observed later. Dosing of ROS and  
315 antioxidative enzymes during exposure would be interesting to further understand implied  
316 processes.

317

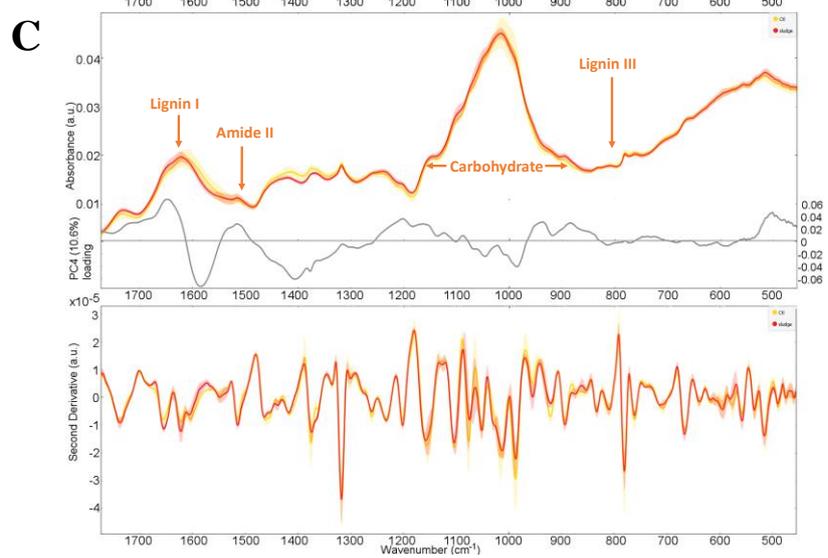
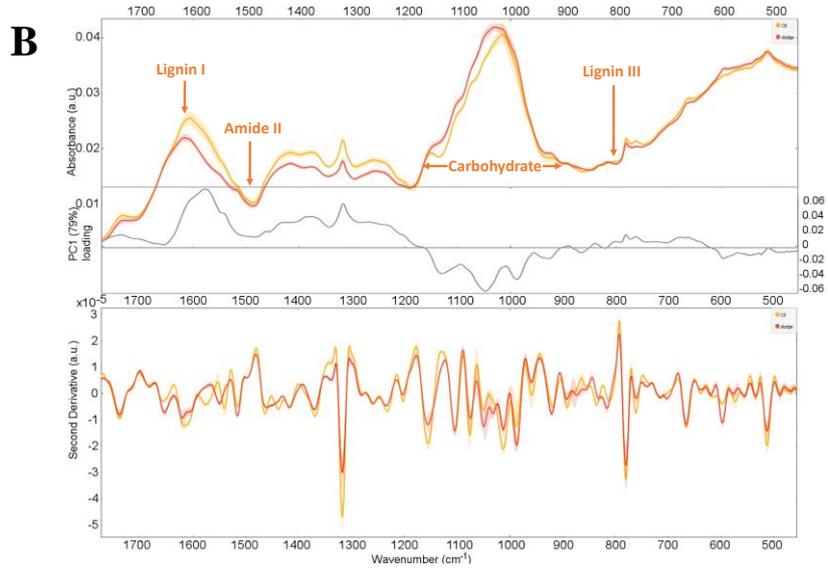
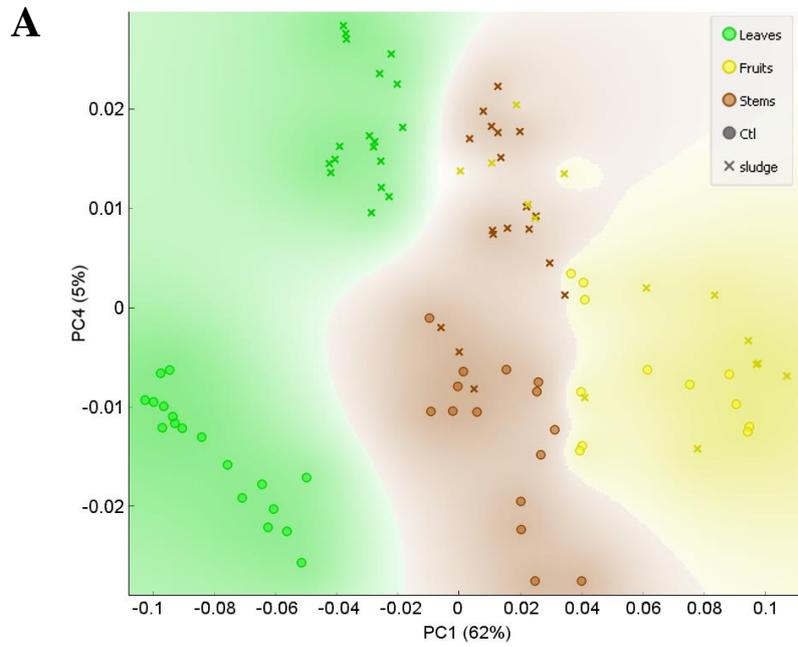
### 318 **3.4. Impact on plant biomacromolecules: FTIR analysis**

319 In this study, FTIR was used to identify changes in specific functional groups in tomato plants  
320 grown in control soil and sludge amended soil (Figure 2A, B, C and Figure S3). During data pre-  
321 processing, on the absorbance spectra, it was noted that most of the variability observed in  
322 the average spectra of each group arose from the carbohydrate/protein/aromatic/saccharide  
323 region (1755-455 cm<sup>-1</sup>), while the PCA on the lipid region (between 3000 and 2800 cm<sup>-1</sup>) failed

324 to separate the groups in this region and the region in-between (2800-1755  $\text{cm}^{-1}$ ) was mainly  
325 background noise.

326 We thus selected the range from 1755 to 455  $\text{cm}^{-1}$ , also known as fingerprint region, for  
327 further processing. After PCA treatment, PC1 (62%) separated clusters corresponding to the  
328 stems, leaves and fruits, while on PC4 (5%) data segregated clearly in two groups (control vs.  
329 sludge amended soil) for leaf samples, to a lesser extent for stem samples but not for fruits  
330 (Figure 2A). These results highlight thus no impact of the sewage sludge treatment on the  
331 biomacromolecules of the edible part of tomato. Also, when comparing the average  
332 absorbance of control and sludge amended soil for the fruits (Figure S3), there is no clear  
333 difference between the spectra, as reflected in the PCA analysis. Excluding the fruits from the  
334 analysis, leaves and stems were further investigated to identify the main spectral differences.  
335 As previously mentioned, the major difference between control and sludge was found in the  
336 leaves. These differences were highlighted with PC1 explaining 79% of the variance between  
337 leaf samples, and changes were evidenced in the second derivative (Figure 2B). The majority  
338 of the differences were associated with spectral bands of carbohydrate (900-1200  $\text{cm}^{-1}$ )  
339 attributed to C-O stretching vibrations and OH deformation vibrations. Differences were also  
340 detected in the region 1480-1180  $\text{cm}^{-1}$  where  $\text{CH}_2$  wagging vibrations occur that may  
341 correspond to aromatic and ring vibrations, followed by changes in the amide, phenolic and  
342 aromatic band contributions (1700-1500  $\text{cm}^{-1}$ )<sup>64</sup> where compounds such as lignin or tannin  
343 (that are mainly aromatic) could be highly contributing. Indeed, the band at 1515  $\text{cm}^{-1}$  is quite  
344 characteristic of lignin<sup>65</sup>.

345



347 **Figure 2: (A) PCA including FTIR spectra from all samples (leaves, stems, fruits) of tomato**  
348 **plants grown for 120 days on control soil and sludge amended soil. FTIR absorbance (upper**  
349 **panel), loadings (in black upper panel) and second derivative (lower panel) spectra of (B)**  
350 **leaves and (C) stems. The shading of the spectra represents the standard deviation.**

351

352 In particular in leaves, the relative intensities of bands 1029, 1050, 1075 and 1144  $\text{cm}^{-1}$  mainly  
353 characteristic of carbohydrates were increased in sludge plants while bands for lignin (666,  
354 760, 780, 1260, 1315, 1370, 1416, 1515, 1540, 1606  $\text{cm}^{-1}$  ) were stronger in control plants.  
355 The spectral region 1200-1610  $\text{cm}^{-1}$  contains many contributions that are characteristic of  
356 tannins<sup>66</sup> and lignin<sup>67</sup>. This result is in agreement with the decrease of tannins observed when  
357 measuring biochemical biomarkers.

358 In stems, the differences were significantly smaller (Figure 2C), to separate the control from  
359 the sludge condition, it was necessary to look into the loadings of the PC4 explaining 10.6%  
360 of the variance of the samples. The major differences occurred in the amide, aromatic regions  
361 (1680-1500  $\text{cm}^{-1}$ ) and in the 1500-1300  $\text{cm}^{-1}$  region, typical of polysaccharides and cellulose.  
362 A decrease at 1417 and 1372  $\text{cm}^{-1}$  and a slight shift from 1614 to 1620  $\text{cm}^{-1}$  was observed in  
363 the sludge condition. This decrease may also be reflecting the changes in concentration of  
364 tannin and lignin but in a less evident manner. Indeed, when looking at the band at 1515  $\text{cm}^{-1}$ ,  
365 no apparent change is observed.

366 With all these observations in mind, we can state that fruits were not affected by the exposure  
367 to the sewage sludge treatment, while some changes were observable in the stems and  
368 leaves. Changes are in the same spectral regions, but the differences are greater in the leaves.  
369 Those results imply that impact of  $\text{TiO}_2$  ENP contamination on food nutritional value of leafy  
370 crop should be investigated more closely. Additionally, impact on fruits cannot be excluded

371 since a study from Servin et al. (2013)<sup>65</sup> evidenced changes in cucumber fruits upon TiO<sub>2</sub> ENP  
372 exposure.

373

### 374 **3.5. Effect on plant elemental concentrations**

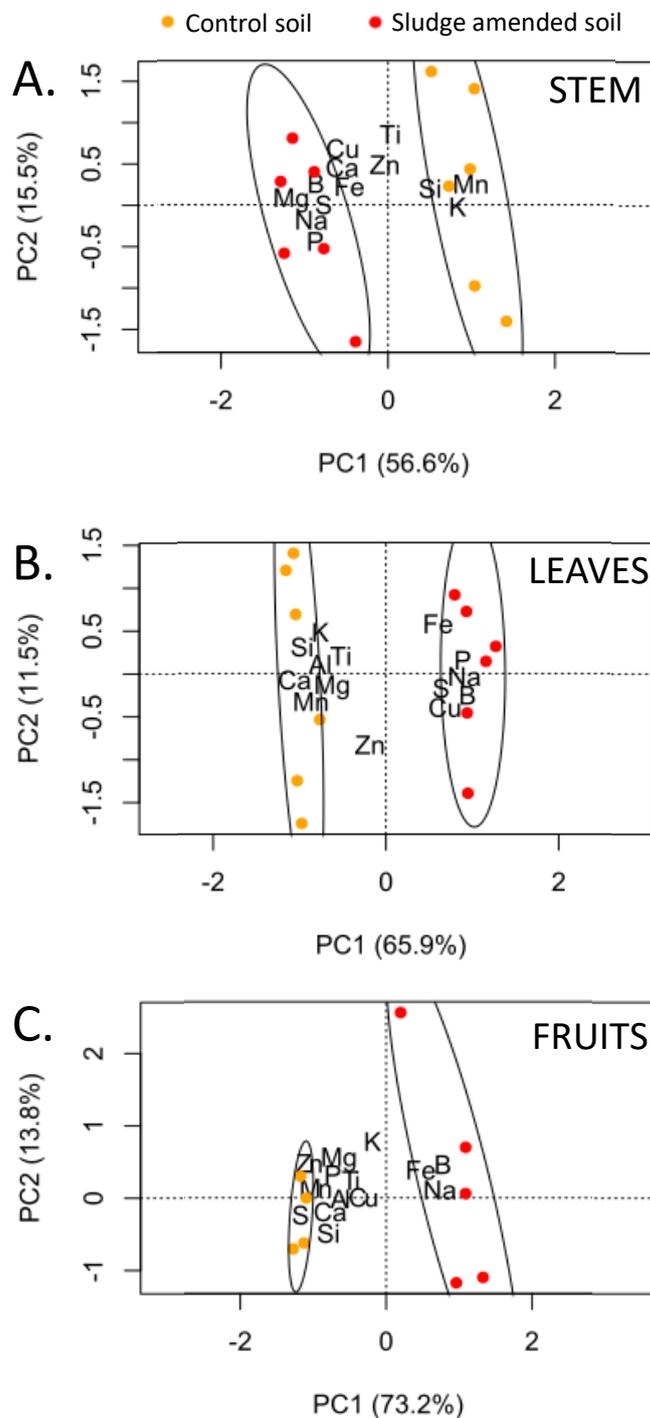
375 Plant macro (S, Ca, Mg, P, K) and microelements (Al, B, Cu, Fe, Mn, Na, Si, Zn) were determined  
376 in the different organs, to assess the impact of sewage sludge amendment on plant ionome  
377 (Figure S4A, B, C). Tomato leaves were again found to be the most impacted plant organ which  
378 is in line with the above discussed FTIR results.

379 Sewage sludge amendment significantly increased the translocation (concentration in the  
380 leaves/concentration in the soil) of Fe, B, and Na in all plant organs (stem, leaves, fruits) while  
381 S, P increased only in leaves and stem (Figure 3A, B, C). Mn and Si translocation was also  
382 consistently lower in sewage sludge exposed plants. Those elements are of primary  
383 importance for plant development. For instance, B is an essential micronutrient influencing  
384 good seed set and fruit development as it plays a vital role in growth of the pollen tube during  
385 flower pollination<sup>68</sup>. Na is a functional nutrient regulating stomatal opening and closing<sup>69</sup>.  
386 Sulfur is also a key component of defense compounds cysteine, glutathione (GSH) and  
387 phytochelatins (PCs) where it appears in the sulfhydryl (-SH or thiol) group. These thiol  
388 compounds synthesised in plants under metal stress represent a very important  
389 detoxification pathways for plant tolerance and survival<sup>70</sup>. Also, according to Amtmann and  
390 Armengaud (2009)<sup>71</sup> changes in nutrient content can impact the overall plant nutritional  
391 quality and taste of crops.

392 Studies in the literature report both sewage sludge<sup>72</sup> and Ti as stimulants of increased nutrient  
393 absorptions in plants<sup>56</sup>. While it is likely that the increase in many elements in plants is due to

394 the effect of the sludge, it can partly also be associated with the presence of TiO<sub>2</sub> functioning  
 395 as promoter of plant nutrition.

396



397

398 **Figure 3: Principal component analysis (PCA) based on elemental translocation factors**

399 **(concentration in the plant organ/concentration in the soil) in (A) stem (B) leaves and (C)**

400 *fruits for tomato plants grown for 120 days on control soil and sludge amended soil, as*  
401 *determined by ICP-OES.*

402

403 In particular, TiO<sub>2</sub> ENPs have been reported to increase P uptake in several studies on  
404 different plant species (lettuce<sup>73</sup>, wheat<sup>74</sup>, tomato<sup>75</sup>). Likewise, Ti has also been associated  
405 with enhancement of Fe uptake<sup>55</sup>. In a hydroponic study of two weeks, nano-TiO<sub>2</sub>  
406 (concentration of 0.5 to 2 g/L) also significantly increased S, Mg and Fe contents in tomato  
407 leaves and roots<sup>75</sup>. Similarly, application of Ti in the form of Tytanit® foliar fertilizer increased  
408 the N, Ca, and Mg content of tomato plants<sup>56</sup>. The decrease in Mn concentration after TiO<sub>2</sub>  
409 ENP application has also been evidenced before<sup>76</sup>.

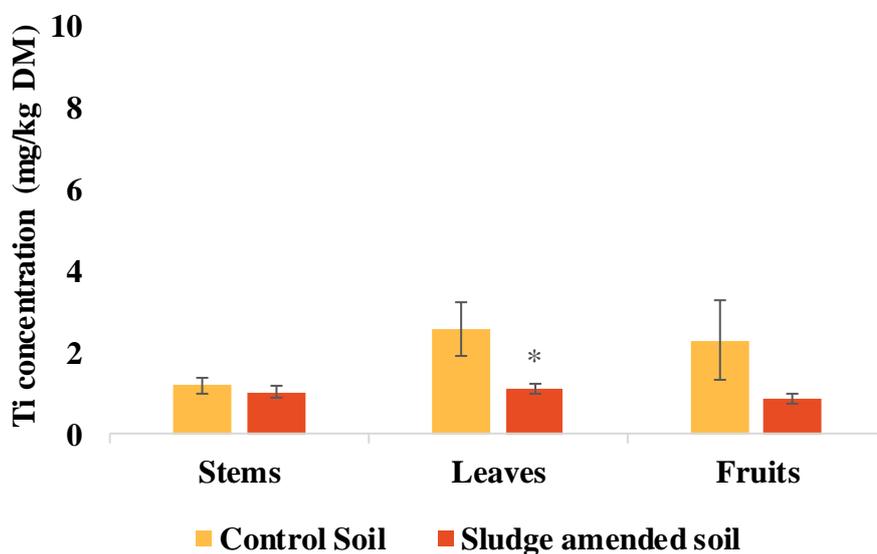
410 Therefore, we assume the elemental impacts to be a cumulative effect of both sludge and  
411 nano- TiO<sub>2</sub>. More studies are needed to distinguish the sludge from the ENP effect, for  
412 example by spiking different concentrations of TiO<sub>2</sub> ENPs in the sewage water before  
413 treatment.

414

### 415 **3.6. Presence of Ti in plant parts**

416 Ti concentrations were  $1.19 \pm 0.51$  and  $1.03 \pm 0.35$  mg/kg in stems,  $2.59 \pm 1.62$  and  $1.14 \pm$   
417  $0.31$  mg/kg in leaves, and  $2.31 \pm 1.67$  and  $0.88 \pm 0.30$  mg/kg in fruits for plants grown on  
418 control soil and sludge amended soil, respectively (Figure 4). While Ti concentration was  
419 similar for both soils Ti uptake was reduced in plant organs in sludge amended soil with  
420 significant reduction in leaves ( $p=0.032$ ). Further  $\mu$ XRF analysis was performed to investigate  
421 Ti distribution in leaf cross sections (Figure S5). However, Ti was below the detection limit in  
422 both samples (description of the experimental set-up and results in SI).

423 Our results are in agreement with the literature that has reported low uptake of Ti based ENPs  
 424 from soil. Du et al. (2011)<sup>30</sup> in their study of 7 months on wheat found no significant difference  
 425 in the Ti content of wheat grain between nano-TiO<sub>2</sub> treated soil (10 g/kg TiO<sub>2</sub>) and control soil  
 426 classified as loamy clay. Burke et al. (2014)<sup>77</sup> also found very low levels of Ti (<2.5 mg/kg) in  
 427 the above ground tissue of soybean and maize plants after six week exposure to 200 mg/kg  
 428 TiO<sub>2</sub> ENPs.  
 429



430  
 431 **Figure 4: Ti concentrations in in the stems, leaves and fruits for tomato plants grown for 120**  
 432 **days on control soil and sludge amended soil. Asterisks indicate significant difference ( $p < 0.05$ )**  
 433 **as indicated by Student T test ( $n=6$ ).**

434  
 435 The lower translocation of Ti in the leaves of plants grown on sewage sludge amended soil  
 436 might be due to the presence of a significantly higher amount of organic matter (+24%)  
 437 consisting of functional groups that readily form complexes with ENPs, compared to the  
 438 control soil, thereby possibly, reducing the bioavailability of Ti. Indeed, studies have reported

439 that pH and organic matter directly influence the bioavailability of ENPs in soil<sup>78</sup>. Similarly, we  
440 evidenced that Ti exhibited no significant root internalization and no upward translocation to  
441 leaves in wheat plants grown in soils with organic matter content above 1.5%.

442 So far there is no defined range for deficiency, sufficiency or toxicity for Ti in plants<sup>79</sup>.  
443 However, Kabata-Pendias and Pendias (2001)<sup>80</sup> suggested that Ti content ranging from 50 to  
444 200 mg kg<sup>-1</sup> in mature leaves of plants could be excessive or toxic while Lyu et al. (2017)<sup>55</sup>  
445 proposed a Ti threshold of 50 mg kg<sup>-1</sup> in leaf tissues. So, based on our results, Ti  
446 concentrations in plant parts were far lower than these proposed concentrations, thus  
447 suggesting no risk of toxicity.

448 Another issue to consider is that nano-TiO<sub>2</sub> exhibit a high potential to adsorb toxic metals on  
449 their surfaces as well as nutrients<sup>81-83</sup>. Over the years, repeated applications of nano-TiO<sub>2</sub>  
450 through sewage sludge might lead to the release of metals sorbed on ENP surface into the  
451 environment becoming readily available for crop plants<sup>10</sup> and the decreased bioavailability of  
452 nutrients such as Ca and Fe<sup>84</sup>. According to Hartmann and Baun (2010)<sup>85</sup> the interaction  
453 between ENPs and toxic compounds in environmental mixtures can either amplify or alleviate  
454 toxicity. But it is still unclear whether the co-contamination of ENPs and metals would lead to  
455 an enhance toxicity of the mixture (Trojan horse effect) or rather a decreased one.

456

457 Our results indicate that sewage sludge amendment containing nano-TiO<sub>2</sub> improved the plant  
458 yield due to its high organic matter and nutrient content and did not lead to significant  
459 changes in the edible part of tomato. However, over time nano-TiO<sub>2</sub> in sewage sludge is  
460 expected to increase while organic matter content may decrease if the sludge application

461 stops. This may affect Ti availability in the long run. Another aspect to take into consideration  
462 is the impact of such amendment on soil bacterial communities along time.

463

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