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

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A combined study of TiO_2 nanoparticle transport and toxicity on microbial nitrifying communities under single and repeated exposures in soil columns

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ABSTRACT

Soils are exposed to nanoparticles (NPs) due to their increasing use in many commercial products. Adverse effects of NPs on soil microorganisms have been reported in several ecotoxicological studies using microcosms. Although repeated exposures are more likely to occur in soils, most of these previous studies were performed as a single exposure to NPs. Contrarily to single contamination, the study of multiple NP contaminations in soils requires the use of specialized setups. Using a soil column experiment, we compared the influence of single and repeated exposures (one, two or three exposures that resulted in the same final concentration) on the transport of titanium dioxide NPs (TiO_2-NPs) through soil and the effect of these different exposure scenarios on the abundance and activity of soil nitrifying microbial communities after a 2-month incubation period. The transport of TiO_2-NPs was very limited under both single and repeated exposures and was highest for the lowest concentration injected during the first application. Significant decreases in nitrification activity and ammonia-oxidizing archaea and bacteria populations were observed only for the repeated exposure scenario (three TiO_2-NP contaminations). These results show that under repeated exposures, the transport of TiO_2-NPs to deep soil layers and groundwater is limited and that a chronic TiO_2-NPs contamination is more harmful for the soil microbiological functioning than a single exposure.

Key-words: TiO_2 nanomaterials, chronic and acute exposure, transport, microbial ecotoxicology, nitrification.

INTRODUCTION

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

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

The aim of this work was to compare the effect of single and repeated NP applications on: (i) the transport of TiO_2-NPs through a soil profile, and (ii) their toxicity to the soil microbial communities by focusing on disruption of the nitrification process which is a critical step in the nitrogen cycle. Nitrification is a crucial microbiological process for soil fertility controlled by the activity of both of ammonia- and nitrite-oxidizers that convert ammonium to nitrate. This key step of the nitrogen cycle is known to be very sensitive to environmental stressors, especially chronic disturbances[12–14]. Therefore, the abundance of ammonia-oxidizing archaea

and bacteria (AOA and AOB, respectively) was selected for monitoring soil toxicity in this study because this endpoint has been proposed as relevant ecological indicators for soil health[15,16]. TiO_2-NPs are the most produced NPs and it has been predicted that they may represent 50 % of all engineered NPs retrieved in soil, especially because of the repeated application of biosolids on agricultural soils[17,18]. TiO_2-NPs are known to be toxic for microbes, even in the absence of light like in conditions encountered in soils, due to a depolarization and loss of membrane integrity translating in a cell osmotic stress[19,20]

Our experimental design, based on saturated soil columns, enabled the study of the fate and impact of both single and repeated TiO_2-NP exposures in soil under more realistic exposure conditions than what can be achieved with classical microcosm experiments. In the context of short-term lab-scale experiment, soil columns were contaminated using three exposure scenarios (one, two, or three applications) resulting in the same total TiO_2-NP concentrations applied to soil columns (372 mg NPs kg^{-1} dry soil). After the last NP application, four layers of the contaminated soil columns corresponding to different depths were collected and incubated separately for 2 months to evaluate the impact of TiO_2-NP exposure on the nitrification process along the soil profile. The nitrification enzyme activity (NEA) and the abundance of AOA and AOB were measured.

MATERIALS AND METHODS

Soil

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

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

TiO_2 nanoparticles

Uncoated titanium dioxide nanoparticles (mixture of anatase (80%) and rutile (20%) crystal structure) were purchased from Sigma Aldrich (St Louis, USA) with at least 99.5 % purity. The mean primary particle size of the TiO_2-NPs was 28.7 ($\pm$ 7.1) nm in powder as measured with a ZEISS Ultra 55 scanning electron microscopy–field emission gun (SEM-FEG) and energy dispersive spectroscopy (EDS) with a SDD detector (BRUKERAXS-30 mm^2).

The size and zeta potential of the TiO_2-NPs were characterized using Dynamic Light Scattering (DLS) in the soil solution used as the background solution in column experiments. The soil solution was prepared following the protocol described by Simonin *et al.*[21]. Briefly, 10 g of soil dispersed in 50 mL of ultrapure water were shaken for 30 min at 180 rpm and 20 °C in a refrigerated incubator shaker (New Brunswick – Eppendorf, Hamburg, Germany). The solutions were then centrifuged for 20 min at 8000*g* and 20 °C (Centrifuge 5804R, Eppendorf, Hamburg, Germany) to eliminate particles larger than 20 nm according to Stokes' law. The supernatants were collected and stored at 4 °C. The resulting soil solution had a pH of 7.1, an ionic strength of 1.6 mM and a dissolved organic carbon (DOC) concentration of 8.9 mg L^{-1}. Measurements were performed in triplicate with three suspensions of TiO_2-NPs (50, 25 and 16.7 mg L^{-1}) after dispersion by ultrasonication (Sonication bath, Bioblock Scientific) for 5 min

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

Exposure of soil in columns to single and repeated TiO_2-NP applications

Three TiO_2-NP suspensions were prepared in soil solution for each exposure scenario: 50 mg L^{-1} for the single exposure experiment, 25 mg L^{-1} for the two-exposures experiment, and 16.7 mg L^{-1} for the three-exposures experiment. For the three exposure scenarios, the soil columns received the same net amount of NPs, corresponding to a final concentration of 372 mg kg^{-1} dry soil, which is high compared to the predicted TiO_2 concentration (1.2 mg kg^{-1} $year^{-1}$) in European soils[18]. The choice of a high input concentration of TiO_2-NPs was made to ensure a titanium concentration in the column effluents that was high enough to be distinguished from the background concentration. Indeed, soils naturally contain very high concentrations of Ti[22] (in average 4000-5000 mg kg^{-1} and 4520 ± 233 mg Ti kg^{-1} dry soil in the studied soil). After saturation and leaching with 100 mL of the soil solution, the column effluents before NP-spiking exhibited an average concentration of 0.33 ± 0.1 mg Ti L^{-1}. Since we observed that TiO_2-NP aggregation and surface charge were significantly different in water and soil solution[21], the suspensions of TiO_2-NPs were prepared in soil solution to achieve a more realistic assessment of the fate and impact of NPs in the soil.

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

concentration in the effluents. During the entire transport experiment, the solutions were injected in the column using a peristaltic pump at a flow rate of 0.1 mL min^{-1}, which corresponds to a Darcy flow of 0.15 cm h^{-1}. After this first step of saturation/leaching, 5 pore volumes (PV) (each pore volume was 6.7 mL) of the TiO_2-NPs suspension (either 50, 25 or 16.7 mg L^{-1}) were injected and then 10 PV of the soil solution free of TiO_2-NPs were immediately injected in the columns.

The single exposure experiment was performed as a one-time injection of 50 mg L^{-1} TiO_2-NPs into six saturated soil columns. Six control columns received the same PV of the soil solution free of TiO_2-NPs.

Repeated exposure experiments were conducted as two or three applications of TiO_2-NPs with 25 and 16.7 mg L^{-1}, respectively in 6 columns for each treatment. Six additional control columns were used for each type of exposure as mentioned above. The successive applications of NPs were separated by a 15-day delay during which columns were maintained horizontally at 28 °C in the dark at constant moisture.

Transport of TiO_2-NPs in soil columns

For each exposure treatment, effluents from a randomly chosen column were continuously sampled into 15 mL centrifuge tubes (1 mL sampled in 10 minutes) using a fraction collector (Gilson, Minipulse 3). Ti concentrations in the spiking suspensions (C_0) and in the effluents (C) were determined using a microwave assisted (Novawave, SCP Science) strong acid extraction method (hydrofluoric acid + nitric acid) and were used to establish dimensionless TiO_2 breakthrough curves (C/C_0). Ti concentrations were measured by inductively coupled plasma - optical emission spectrophotometry (ICP-OES; Varian 700-ES, Varian Inc. Scientific 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.*[23], TiO_2-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^{-1} dry soil) compared to the TiO_2-NP input concentration (372
167 mg kg^{-1}). Indeed, the standard error of the soil total Ti content measurements (233 mg kg^{-1}
168 dry soil) was too close to the TiO_2-NP concentration added to the soil to expect reliable values
169 of TiO_2-NPs concentration in the different soil layers. Thus, our estimation of the final TiO_2-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_2-NPs on the nitrification process along the soil profile**

174 The impact of TiO_2-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

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

DNA extraction and abundance of ammonia-oxidizers

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

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

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

Nitrification enzyme activity

NEA was determined according to the protocol described by Dassonville *et al.* [25]. Sub-samples of fresh soil (1.5 g equivalent dry weight) were incubated with 3 mL of a solution of $(NH_4)_2SO_4$ in order to reach nitrogen-as-ammonia ($N\text{-}NH_4^+$) concentrations of 50 µg g^{-1} dry soil. Distilled water was added to each sample to achieve 12 mL of total liquid volume in glass plasma flasks. The flasks were sealed with Parafilm® and incubated at 28 °C under 180 rpm constant agitation. During the incubation, 1 mL of soil slurry was sampled at 2 h, 4 h, 6 h, 8 h and 10 h, filtered (0.2 µm pore size) and transferred in vials stored at -20 °C. The analysis of NO_3^- concentrations was performed by ionic chromatography (DX120, Dionex, Salt Lake City, USA) equipped with a 4 mm × 250 mm column (IonPac AS9 HC). NEA was expressed as µg nitrogen-as-nitrate ($N\text{-}NO_3^-$) h^{-1} g^{-1} dry soil.

Statistical analysis

All results are presented as means (± standard error). A two-way analysis of variance (ANOVA) and *post-hoc* Tukey's honest significant difference (HSD) were performed to test the effect of TiO_2-NP exposures and soil depth on the nitrifying activity and ammonia-oxidizer abundance for each type of exposure scenario separately. Data were log-transformed prior to analysis when necessary to ensure conformity with the assumptions of normality and homogeneity of variances. T-tests were conducted to compare the aggregation and zeta potential of TiO_2-NPs in the different spiking suspensions. All statistical analyses were carried out using the R statistical software 2.13.2[26].

RESULTS

Characteristics of TiO_2-NPs in the spiking soil solutions

TiO_2-NPs were characterized in the suspensions used to contaminate soil columns under the three exposure scenarios. The aggregation of TiO_2-NPs increased with the concentration (Table 2). At the lowest concentrations applied three times successively in soil columns (16.7 mg L^{-1}), the average TiO_2-NP hydrodynamic diameter was 111.2 (± 10.1) nm, while at the highest concentration used for the single application (50 mg L^{-1}), the hydrodynamic diameter was 153.9 (± 9.97) nm.

The surface charge of TiO_2-NPs assessed through zeta potential measurements (electrophoretic mobility) were extremely close for the three suspension concentrations (-15.4 to -16.6 mV; Table 2), even if it was significantly lower in the 25 mg L^{-1} suspension (Table 2).

Effect of TiO_2-NP concentration and of the number of applications on their mobility in soil

The experimental design enabled the study of the influence of TiO_2-NPs concentration on their transport through soil after single exposures at 16.7, 25 or 50 mg L^{-1} and after repeated exposures consisting of two or three applications of NPs in suspensions containing 25 and 16.7 mg L^{-1}, respectively (Table 3).

The mobility of TiO_2-NPs decreased with increasing TiO_2-NP concentration (Table 3). For an injected concentration of 16.7 mg L^{-1}, 10.66 % of TiO_2-NPs were recovered in the effluents, whereas only 4.95 and 2.19 % were recovered when applying the suspensions containing 25 and 50 mg L^{-1}, respectively.

In the columns that received two applications of NPs, the mobility of TiO_2-NPs was greatly reduced compared to the mobility measured during the first application (Table 3). Only 0.9 % of the added TiO_2-NPs were recovered in the effluents for both 25 and 16.7 mg L^{-1} exposure concentrations (Table 3). For the third NP application at 16.7 mg L^{-1}, the mobility of TiO_2-NPs was almost negligible (0.2%; Table 3).

We calculated the final TiO_2-NP concentration retained in the column on the basis of the mass recovery calculations in the effluents after background subtraction (Table 3). Although the same theoretical TiO_2-NPs concentration of 372 mg kg^{-1} was applied to the soil, the final NPs retained concentration differed in the three exposure scenarios (Table 3). The highest final concentration of TiO_2-NPs (364 mg kg^{-1}) was measured in the columns exposed to a single application at 50 mg L^{-1}. In the columns receiving two applications at 25 mg L^{-1}, the final TiO_2-NPs concentration was estimated at 350 mg kg^{-1}, which corresponds to a decrease of 6 % compared to the single exposure. After three applications at 16.7 mg L^{-1}, only 328 mg kg^{-1} of TiO_2-NPs were retained in the soil, which corresponds to a decrease of 12 % and 6 %, compared to the single and dual exposure scenarios, respectively.

Effect of single and repeated NP contaminations on nitrification enzyme activity and ammonia-oxidizer abundance

After two months of incubation following the last application of NPs to soil columns, NEA, AOA and AOB abundances were measured. When considering the whole soil column (i.e. the four soil layers), the single exposure (i.e. single application at 50 mg L^{-1}) resulted in a significant increase of the AOA abundance (Figure 1; Table 4) but no effect was observed on AOB abundance or NEA (Figure 1; Table 4). After two successive NP applications at 25 mg L^{-1}, neither the NEA nor the ammonia-oxidizer abundances were affected by TiO_2-NPs

contamination (Figure 1, Table 4). In contrast, NEA, AOA and AOB abundance were significantly decreased after three repeated applications of TiO_2-NPs at 16.7 mg L^{-1} (Figure 1; Table 4).

A significant effect of soil depth on NEA was only observed after two and three exposures (P = 0.02 and P = 0.003, respectively; Table 4). After two soil exposures to NPs, the NEA differed significantly according to soil depth, especially at 2–4 cm (Figure 2). After three exposures, NEA was significantly more reduced by TiO_2-NPs in the first 2 cm than in the other soil layers (Figure 2). No significant statistical interaction between TiO_2-NPs contamination and soil depth was found (Table 4).

DISCUSSION

Consequences of single and repeated exposures on TiO_2-NPs transport in soil

Our experimental design based on soil columns dynamic contamination enabled coupled mobility and toxicity studies of TiO_2-NPs under single and repeated exposures at different input concentrations. The results showed that the transport of TiO_2-NPs is low (90 to 99% of retention) and influenced by the concentration injected in the soil columns. After a single application, TiO_2-NP retention in soil increased with increasing injected concentrations. Similar behavior of silica nanoparticles has been reported in sand columns by Vitorge *et al.*[27], who showed that NPs mobility increases at low concentration regimes. These results can be explained by the increase of TiO_2-NP aggregation with increasing concentration, which can favor the filtration, deposition and straining of these colloids[7,28]. Although the significant difference of TiO_2-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_2-NPs in soil reported contrasting results.
295 Nickel et al. (2015)[23] observed no transport using an input concentration of 1 g TiO_2-NPs L^{-1},
296 while significant transport was recorded in different soils exhibiting low clay contents and
297 ionic strengths with several applied concentrations of TiO_2-NPs[9]. Here, we show that although
298 low, the transport of TiO_2-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_2-NPs will be likely very low, in
302 agreement with a classical blocking mechanism typically observed at high colloidal
303 concentrations[27,28]

304 For repeated exposure in soil columns, an extremely low mobility of TiO_2-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_2-NPs along the soil profile could not be measured
307 without specific labeling[23]. However, these results observed in only 8 cm long columns suggest
308 that in soils repeatedly exposed to TiO_2-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_2-NPs transport in longer soil columns and
312 in undisturbed soil cores.

313

Deleterious effects on nitrification after repeated exposure

Two months after the last application of TiO_2-NPs to the soil columns, significant decreases in NEA and ammonia-oxidizer abundances were observed only in soil samples exposed three times to the lowest TiO_2-NPs concentration (16.7 mg L^{-1}). In this case, the final TiO_2-NPs concentration in soil was slightly lower than in the two other exposure scenarios. Surprisingly, we have observed an increase of AOA abundance in the single exposure scenario but without significant changes on NEA. A possible explanation is that a shift occurred in the AOA community and that AOA populations with lower nitrifying activity were stimulated in these conditions. We show in this short-term experiment that soil repeated exposure to TiO_2-NPs has a more deleterious impact on soil microbial community and soil functioning than a single exposure. This result highlights that the nitrifying population did not become more resistant and resilient to TiO_2-NPs after multiple exposures. On the contrary, the nitrification process and ammonia-oxidizers became more sensitive, probably because organisms stressed by the previous exposures to NPs had less energy to cope with additional disturbances and/or because of a decrease or a shift of soil microbial diversity[29]. It is well known that a microbial community can become tolerant to various stressors over time through the selection of more tolerant populations and/or the dispersal of specific resistances *via* mobile genetic elements[14,29,30]. Chronic exposures to pollutants occurring over a longer term will likely have less consequences on nitrification because of potential microbial community adaptation over time, as it was observed by Mertens *et al.*[31] after 2 years of exposure to zinc pollution. To gain a better knowledge of NP impact on soil functioning under chronic contamination, it would be necessary to study the effects on microbial activity, abundance and diversity after each exposure and before each new exposure, in order to monitor the resistance and resilience of the microbial community in the long term. This approach could lead to new insights into

sensitive and tolerant microbial species among nitrifiers and thus on TiO_2-NP toxicity and adaptation mechanisms in soil.

After three NP applications, it is likely that most of TiO_2-NPs were retained close to the inlet of the column through filtration and/or straining. Although a TiO_2 concentration gradient can be expected along the soil profile according to the filtration theory[32], no significant effect of depth on ammonia-oxidizer abundance was observed. Nitrification was significantly more diminished in the 0–2 cm and 6–8 cm layers than in the middle 2–4 and 4–6 cm soil layers. With common soluble pollutants, the general assumption is that the highest concentrations induce the most deleterious effects. In the case of NPs, concentration effects are more complex because of aggregation processes and thus the reactivity, bioavailability and toxicity of these contaminants can vary in function of the spiked concentration[21,33–35]. Such changes in NPs aggregation with concentration are likely to induce nonlinear dose-effect relationships[36]. Our results suggest that microbial communities may be affected by TiO_2-NPs at the depths of preferential NP accumulation (top of the soil column) but also at deeper depths likely reached by NPs at lower concentration (bottom of the soil column).

The multidisciplinary approach developed in this study enabled the comparison of the impact of single and repeated TiO_2-NPs exposures in water-saturated soil columns. The transport of TiO_2-NPs in the studied silty-clay soil was evidenced but was limited (over 90% retention) for both single and repeated applications in 8 cm long columns. TiO_2-NPs mobility was also shown to increase at low NPs concentrations indicating an aggregation-controlled process. Considering these results, the transport of TiO_2-NPs to deeper soil layers or groundwater appears limited. Although the repeat exposures resulted in lower final TiO_2-NP concentrations in soil, the impact was greater on the soil microbial community and on soil functioning (NEA)

compared to the single exposure condition. Future studies should address the fate and effects of TiO_2-NPs chronically added to soils in biosolid amendments, as it is one of the most relevant routes by which NPs enter the soil environment[37]. Chronic exposure in short and long term experiments representing realistic scenarios of soil exposure to NPs are urgently needed to study the resistance and resilience of soil microbial communities to these emerging contaminants and their consequences on soil fertility.

TABLE CAPTIONS

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

Table 2 Size and zeta potential of TiO_2-NPs in soil solution for the 3 tested concentrations measured with a Nano ZS (Malvern). Means and standard errors are presented (n = 3). Values labeled with the same letter do not differ at $P < 0.05$.

Table 3 TiO_2 relative mass recoveries in column effluents for the three applied NPs concentrations in the different exposure scenarios (one, two, or three applications). The final TiO_2-NP concentration retained in the column was calculated from the relative mass recovery of TiO_2-NPs in the effluents. The presented values have been corrected by the Ti background concentration in the effluents measured before NP application (0.33 mg Ti L^{-1}).

Table 4 P-values from an ANOVA analysis of the effects of TiO_2-NPs, soil depth and their interaction on nitrification enzyme activity, AOA and AOB abundances for each exposure scenario. Values in bold are significant at $P < 0.05$.

FIGURE CAPTIONS

Figure 1 Effect of TiO_2-NPs on nitrification enzyme activity (NEA), AOA and AOB abundances in whole columns (average of the 4 soil depths). Data are expressed as the percentage of change relative to the control treatment for each exposure. Standard errors are presented (n = 6). Significant effects are indicated (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

Figure 2 Effect of TiO_2-NPs on nitrification enzyme activity (NEA) measured in the four soil column layers (depths 0-2 cm; 2-4 cm; 4-6 cm; 6-8 cm) after one, two or three soil exposures. Data are expressed as percentage of change from the control treatment for each exposure condition. Standard errors are presented (n = 6). For each exposure scenario, bars labeled with the same letter do not differ at $P < 0.05$.

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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.

(13) Broos, K.; Mertens, J.; Smolders, E. Toxicity of heavy metals in soil assessed with various soil microbial and plant growth assays: A comparative study. *Environ. Toxicol. Chem.* **2005**, *24* (3), 634–640.
(14) Bissett, A.; Brown, M. V.; Siciliano, S. D.; Thrall, P. H. Microbial community responses to anthropogenically induced environmental change: towards a systems approach. *Ecol. Lett.* **2013**, *16*, 128–139.
(15) Schloter, M.; Dilly, O.; Munch, J. C. Indicators for evaluating soil quality. *Agric. Ecosyst. Environ.* **2003**, *98* (1–3), 255–262.
(16) Wessén, E.; Hallin, S. Abundance of archaeal and bacterial ammonia oxidizers – Possible bioindicator for soil monitoring. *Ecol. Indic.* **2011**, *11* (6), 1696–1698.
(17) Keller, A. A.; McFerran, S.; Lazareva, A.; Suh, S. Global life cycle releases of engineered nanomaterials. *J. Nanoparticle Res.* **2013**, *15* (6), 1–17.
(18) Sun, T. Y.; Gottschalk, F.; Hungerbühler, K.; Nowack, B. Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. *Environ. Pollut.* **2014**, *185*, 69–76.
(19) Sohm, B.; Immel, F.; Bauda, P.; Pagnout, C. Insight into the primary mode of action of TiO2 nanoparticles on Escherichia coli in the dark. *Proteomics* **2015**, *15* (1), 98–113.
(20) Erdem, A.; Metzler, D.; Cha, D. K.; Huang, C. P. The short-term toxic effects of TiO2 nanoparticles toward bacteria through viability, cellular respiration, and lipid peroxidation. *Environ. Sci. Pollut. Res.* **2015**, *22* (22), 17917–17924.
(21) Simonin, M.; Guyonnet, J. P.; Martins, J. M. F.; Ginot, M.; Richaume, A. Influence of soil properties on the toxicity of TiO2 nanoparticles on carbon mineralization and bacterial abundance. *J. Hazard. Mater.* **2015**, *283*, 529–535.
(22) Aubert, H.; Pinta, M. *Trace Elements in Soils*; Elsevier, 1980.
(23) Nickel, C.; Gabsch, S.; Hellack, B.; Nogowski, A.; Babick, F.; Stintz, M.; Kuhlbusch, T. A. Mobility of coated and uncoated TiO 2 nanomaterials in soil columns–Applicability of the tests methods of OECD TG 312 and 106 for nanomaterials. *J. Environ. Manage.* **2015**, *157*, 230–237.
(24) Treusch, A. H.; Leininger, S.; Kletzin, A.; Schuster, S. C.; Klenk, H.-P.; Schleper, C. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ. Microbiol.* **2005**, *7* (12), 1985–1995.
(25) Dassonville, N.; Guillaumaud, N.; Piola, F.; Meerts, P.; Poly, F. Niche construction by the invasive Asian knotweeds (species complex Fallopia): impact on activity, abundance and community structure of denitrifiers and nitrifiers. *Biol. Invasions* **2011**, *13* (5), 1115–1133.
(26) R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria 2015.
(27) Vitorge, E.; Szenknect, S.; Martins, J. M. F.; Gaudet, J.-P. Size- and concentration-dependent deposition of fluorescent silica colloids in saturated sand columns: transport experiments and modeling. *Environ. Sci. Process. Impacts* **2013**, *15* (8), 1590–1600.
(28) Solovitch, N.; Labille, J.; Rose, J.; Chaurand, P.; Borschneck, D.; Wiesner, M. R.; Bottero, J.-Y. Concurrent Aggregation and Deposition of TiO2 Nanoparticles in a Sandy Porous Media. *Environ. Sci. Technol.* **2010**, *44* (13), 4897–4902.
(29) Griffiths, B. S.; Philippot, L. Insights into the resistance and resilience of the soil microbial community. *FEMS Microbiol. Rev.* **2013**, *37* (2), 112–129.

(30) Allison, S. D.; Martiny, J. B. H. Resistance, resilience, and redundancy in microbial communities. *Proc. Natl. Acad. Sci.* **2008**, *105* (Supplement 1), 11512–11519.
(31) Mertens, J.; Broos, K.; Wakelin, S. A.; Kowalchuk, G. A.; Springael, D.; Smolders, E. Bacteria, not archaea, restore nitrification in a zinc-contaminated soil. *ISME J.* **2009**, *3* (8), 916–923.
(32) Huber, N.; Baumann, T.; Niessner, R. Assessment of Colloid Filtration in Natural Porous Media by Filtration Theory. *Environ. Sci. Technol.* **2000**, *34*, 3774–3779.
(33) Choi, O. K.; Hu, Z. Q. Nitrification inhibition by silver nanoparticles. *Water Sci. Technol.* **2009**, *59* (9), 1699.
(34) Simon-Deckers, A.; Loo, S.; Mayne-L'hermite, M.; Herlin-Boime, N.; Menguy, N.; Reynaud, C.; Gouget, B.; Carrière, M. Size-, Composition- and Shape-Dependent Toxicological Impact of Metal Oxide Nanoparticles and Carbon Nanotubes toward Bacteria. *Environ. Sci. Technol.* **2009**, *43* (21), 8423–8429.
(35) Menard, A.; Drobne, D.; Jemec, A. Ecotoxicity of nanosized TiO2. Review of in vivo data. *Environ. Pollut.* **2011**, *159* (3), 677–684.
(36) Bernhardt, E. S.; Colman, B. P.; Hochella, M. F.; Cardinale, B. J.; Nisbet, R. M.; Richardson, C. J.; Yin, L. An ecological perspective on nanomaterial impacts in the environment. *J. Environ. Qual.* **2010**, *39* (6), 1954–1965.
(37) Pradas del Real, A. E.; Castillo-Michel, H.; Kaegi, R.; Sinnet, B.; Magnin, V.; Findling, N.; Villanova, J.; Carrière, M.; Santaella, C.; Fernández-Martínez, A.; et al. Fate of Ag-NPs in Sewage Sludge after Application on Agricultural Soils. *Environ. Sci. Technol.* **2016**, *50* (4), 1759–1768.