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A combined study of TiO₂ 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 (TiO₂-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 TiO₂-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 TiO₂-NP contaminations). These results show that under repeated exposures, the transport of TiO₂-NPs to deep soil layers and groundwater is limited and that a chronic TiO₂-NPs contamination is more harmful for the soil microbiological functioning than a single exposure.

Key-words: TiO₂ 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 fertility¹⁻³. In most of these studies, soils were exposed to single homogeneous applications of NPs into microcosms³. 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 nanopesticides⁴⁻⁶ and may be leached to further depths through soil porosity⁷. Therefore in contaminated soils NPs are heterogeneously distributed, especially along vertical soil profiles⁸. 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 theory⁹⁻¹¹ and also by NP properties, such as size and/or surface charge⁷.

To date, only a few studies were performed on repeated or chronic NP exposure in soil¹². 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 TiO₂-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 disturbances¹²⁻¹⁴. 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}. TiO₂-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}. TiO₂-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 TiO₂-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 TiO₂-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 TiO₂-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.

TiO₂ 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 TiO₂-NPs was 28.7 (± 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 mm²).

The size and zeta potential of the TiO₂-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 8000g 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 TiO₂-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 TiO₂-NP applications

Three TiO₂-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 TiO₂ concentration (1.2 mg kg⁻¹ year⁻¹) in European soils¹⁸. The choice of a high input concentration of TiO₂-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 Ti²² (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 TiO₂-NP aggregation and surface charge were significantly different in water and soil solution²¹, the suspensions of TiO₂-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

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

Consistently with Nickel *et al.*²³, TiO₂-NP concentrations retained in the different soil layers after contamination could not be accurately measured due to the naturally high titanium content of the soil (4520 mg Ti kg⁻¹ dry soil) compared to the TiO₂-NP input concentration (372 mg kg⁻¹). Indeed, the standard error of the soil total Ti content measurements (233 mg kg⁻¹ dry soil) was too close to the TiO₂-NP concentration added to the soil to expect reliable values of TiO₂-NPs concentration in the different soil layers. Thus, our estimation of the final TiO₂-NP concentration retained in soil is based on the measured Ti concentrations (mass balance) in the column effluents and not a direct measurement of the Ti concentration in soil.

Impact of TiO₂-NPs on the nitrification process along the soil profile

The impact of TiO₂-NPs on soil nitrification activity and ammonia-oxidizing microorganisms abundance as a function of soil depth was studied 2 months after the last exposure to NPs. Immediately after the last exposure, soil columns were sliced in four layers according to the distance from the inlet (0–2 cm, 2–4 cm, 4–6 cm and 6–8 cm). Each slice was then placed into 60 mL glass flasks and incubated at constant moisture for 2 months at 28 °C in the dark. This experimental design resulted in 144 samples: 6 column treatments (three exposure scenarios and three control treatments) x 4 depths x 6 replicates. At the end of the incubation time, 1.5 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 10⁷–10² gene copies number of an in-house plasmid containing cloned bacterial (*Nitrosomonas europaea*, GenBank accession number:L08050) and archaeal (54d9 fosmid fragment²⁴) *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 $(\text{NH}_4)_2\text{SO}_4$ in order to reach nitrogen-as-ammonia (N-NH_4^+) concentrations of $50 \mu\text{g g}^{-1}$ 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 $\times$ 250 mm column (IonPac AS9 HC). NEA was expressed as μg nitrogen-as-nitrate (N-NO_3^-) $\text{h}^{-1} \text{g}^{-1}$ dry soil.

Statistical analysis

All results are presented as means ($\pm$ 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 2.13.2²⁶.

RESULTS

Characteristics of TiO₂-NPs in the spiking soil solutions

TiO₂-NPs were characterized in the suspensions used to contaminate soil columns under the three exposure scenarios. The aggregation of TiO₂-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 TiO₂-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 TiO₂-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 TiO₂-NP concentration and of the number of applications on their mobility in soil

The experimental design enabled the study of the influence of TiO₂-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 TiO₂-NPs decreased with increasing TiO₂-NP concentration (Table 3). For an injected concentration of 16.7 mg L⁻¹, 10.66 % of TiO₂-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 TiO₂-NPs was greatly reduced compared to the mobility measured during the first application (Table 3). Only 0.9 % of the added TiO₂-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 TiO₂-NPs was almost negligible (0.2%; Table 3).

We calculated the final TiO₂-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 TiO₂-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 TiO₂-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 TiO₂-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 TiO₂-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 TiO₂-NPs

contamination (Figure 1, Table 4). In contrast, NEA, AOA and AOB abundance were significantly decreased after three repeated applications of TiO₂-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 TiO₂-NPs in the first 2 cm than in the other soil layers (Figure 2). No significant statistical interaction between TiO₂-NPs contamination and soil depth was found (Table 4).

DISCUSSION

Consequences of single and repeated exposures on TiO₂-NPs transport in soil

Our experimental design based on soil columns dynamic contamination enabled coupled mobility and toxicity studies of TiO₂-NPs under single and repeated exposures at different input concentrations. The results showed that the transport of TiO₂-NPs is low (90 to 99% of retention) and influenced by the concentration injected in the soil columns. After a single application, TiO₂-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 TiO₂-NP aggregation with increasing concentration, which can favor the filtration, deposition and straining of these colloids^{7,28}. Although the significant difference of TiO₂-NP aggregation between the three concentrations tested in soil solution (42

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

Previous studies on the mobility of uncoated TiO₂-NPs in soil reported contrasting results. Nickel et al. (2015)²³ observed no transport using an input concentration of 1 g TiO₂-NPs L⁻¹, while significant transport was recorded in different soils exhibiting low clay contents and ionic strengths with several applied concentrations of TiO₂-NPs⁹. Here, we show that although low, the transport of TiO₂-NPs can occur after a single exposure despite the fine texture of the studied soil (39 % clay and 51 % loam), especially at the lowest concentration regime, which is more likely to be observed in natural soils. In the case of an accidental spill for which high NPs concentrations can be expected, the mobility of TiO₂-NPs will be likely very low, in agreement with a classical blocking mechanism typically observed at high colloidal concentrations^{27,28}

For repeated exposure in soil columns, an extremely low mobility of TiO₂-NPs was observed (< 1%) and no concentration effect was observed contrarily to what was observed with the single exposures. The distribution of TiO₂-NPs along the soil profile could not be measured without specific labeling²³. However, these results observed in only 8 cm long columns suggest that in soils repeatedly exposed to TiO₂-NPs, these contaminants would accumulate in the top soil, where, coincidentally, most of the biological activity occurs. In these conditions, it is likely that NPs transport to deeper soil layers and groundwater is limited. In order to confirm this assumption future studies are needed to assess TiO₂-NPs transport in longer soil columns and in undisturbed soil cores.

Deleterious effects on nitrification after repeated exposure

Two months after the last application of TiO₂-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 TiO₂-NPs concentration (16.7 mg L⁻¹). In this case, the final TiO₂-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 TiO₂-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 TiO₂-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 diversity²⁹. 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 TiO₂-NP toxicity and adaptation mechanisms in soil.

After three NP applications, it is likely that most of TiO₂-NPs were retained close to the inlet of the column through filtration and/or straining. Although a TiO₂ concentration gradient can be expected along the soil profile according to the filtration theory³², 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 relationships³⁶. Our results suggest that microbial communities may be affected by TiO₂-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 TiO₂-NPs exposures in water-saturated soil columns. The transport of TiO₂-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. TiO₂-NPs mobility was also shown to increase at low NPs concentrations indicating an aggregation-controlled process. Considering these results, the transport of TiO₂-NPs to deeper soil layers or groundwater appears limited. Although the repeat exposures resulted in lower final TiO₂-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 TiO₂-NPs chronically added to soils in biosolid amendments, as it is one of the most relevant routes by which NPs enter the soil environment³⁷. 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 TiO₂-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 TiO₂ relative mass recoveries in column effluents for the three applied NPs concentrations in the different exposure scenarios (one, two, or three applications). The final TiO₂-NP concentration retained in the column was calculated from the relative mass recovery of TiO₂-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 TiO₂-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 TiO₂-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 TiO₂-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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UMR5557-USC1364, Lyon) and quantitative PCR at the DTAMB platform (IFR 41, University Lyon 1). LTHE is part of the Labex OSUG@2020 (ANR10 LABX56).

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