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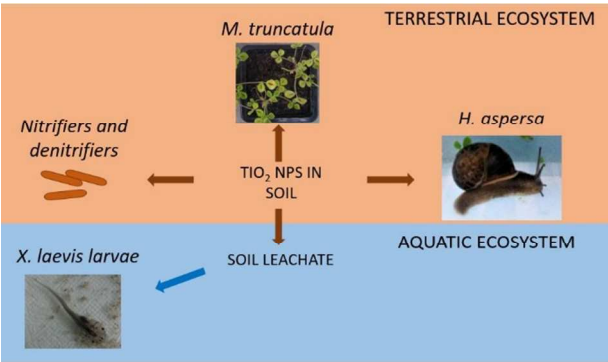
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Transfer and Ecotoxicity of Titanium Dioxide Nanoparticles in Terrestrial and Aquatic Ecosystems: A Microcosm Study

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Graphical abstract



ABSTRACT: With the advancement in nanotechnology, particularly the use of TiO₂ nanoparticles (NPs), there is a need to study their release into the environment and assess the related risk in an environmentally relevant contamination scenario. In the present study, the transfer and toxicity of TiO₂ NPs in microcosms mimicking a terrestrial and an aquatic ecosystems were evaluated. The contaminated soil was prepared by spiking natural soils and used as the basis for all exposure systems including preparation of soil leachates for amphibian exposure. Results demonstrated significant reductions in bacterial (-45%) and archaeal (-36%) nitrifier abundance; significant translocation of Ti to *M. truncatula* leaves (+422%); significant reductions in plant height (-17%), number of leaves (-29%), and aboveground biomass (-53%); non-significant Ti uptake in snail foot and viscera, and excretion in feces; and genotoxicity to *X. laevis* larvae (+119% micronuclei). Our study highlights a possible risk of engineered TiO₂ NPs in the environment in terms of trophic transfer and toxicity in both terrestrial and aquatic environments.

INTRODUCTION

Nanoparticles (NPs) are particles with at least one dimension smaller than 100 nm.¹ Compared with their micro/macro particle counterparts, NPs have a larger surface area over volume ratio,² and number of surface atoms, which results in increased surface energy and reactivity.³ The understanding of their unique properties has rendered them indispensable in several fields of science and technology. Although not all NPs may be toxic, because they are manufactured in a variety of sizes and shapes for use in a multitude of products and industrial processes,⁴ they could pose different potential hazards.^{5,6} Despite the recommendation by the National Organic Standards Board in 2010, products containing NPs are still being manufactured worldwide.⁴ It is therefore necessary to understand the risks associated with their release in the environment.

Among the most widely used NPs are silver and titanium dioxide (TiO₂) NPs.⁷ TiO₂ NPs are commonly known for their photocatalytic applications and used as semiconductors,⁸ additives in food,⁹ and in cosmetic products.¹⁰ Ti is also a widespread element in the environment, with concentrations in soils ranging for instance from 43 mg kg⁻¹ in sand to 1671 mg kg⁻¹ in clayey soil.¹¹ The concern, however, is the increase in this background concentration beyond an environmentally safe threshold, in addition to the potential toxicity of these NPs, on their own, due to the differences in their properties and behavior when compared with natural Ti. Ultimately these NPs will reach the environment.¹² In wastewater treatment plants, NPs partition between treated water and sludge effluents. Through the discharge of treated water, NPs can reach aquatic ecosystems. But the majority (99%)¹³ that remains in sewage sludge is dispersed into agricultural fields as fertilizer.¹⁴ Runoff from these polluted soils can in turn reach water bodies. A recent study by Sun *et al.*¹⁵ predicted the concentrations of TiO₂ NPs in surface waters to be ~ 2.17 µg L⁻¹, in sludge 1.60 g kg⁻¹, and in sludge-treated soils 61.10 mg kg⁻¹. An

accumulation in both terrestrial and aquatic organisms could be carried upward into the food chain by primary and secondary consumption routes.

Negative effects have already been noticed, as a result of NP exposure, in terrestrial and aquatic organisms at the single-organism scale,¹⁶ but the study of NP effects in a simplified food web is not very common. Suggestions have been made that certain NPs can bioaccumulate and possibly bio-magnify in the trophic chain.^{17,18} Yeo and Nam¹⁹ compared the bioaccumulation of TiO₂ NPs and carbon nanotubes in a paddy ecosystem, and observed a bioaccumulation from plankton to ricefish. In a microcosm containing soil, lettuce, crickets, and lizards, Servin *et al.*²⁰ demonstrated that the availability of CuO NPs to terrestrial organisms can be modified by weathering. Using leaf litter decomposition as a functional marker for ecotoxicity in freshwater mesocosms, Bour *et al.*²¹ showed that this ecosystem function was not impacted by CeO₂ NP exposure, in line with the absence of significant differences in numbers, growth, or development of chironomid larvae. However, studies are lacking in terms of a real-life scenario where transfer of TiO₂ NPs occurs from the soil into the water compartment and their impact on, and uptake by, relevant organisms of these environments.

In the present study, the aim was to combine two microcosms mimicking both terrestrial (soil, soil microbial communities, alfalfa plants, and snails) and aquatic (soil leachates and amphibian larvae) compartments to (a) determine whether there is a transfer of Ti within and between these two compartments and (b) evaluate the toxicity of TiO₂ NPs to soil microbial communities (total abundance and abundance of functional guilds), plant (height, number of leaves, aboveground biomass, chlorophyll), snail (mortality, avoidance behavior), and amphibian larvae (mortality, growth, and genotoxicity). Our study contributes valuable data in terms of the impact of TiO₂

NPs on environmentally relevant bioindicators of pollution and provides an insight into implications for food safety and ecotoxicological risk assessment.

1. MATERIALS AND METHODS

1.1. TiO₂ NP characterization and suspension preparation

TiO₂ NPs used in this study were provided by Sigma-Aldrich (P25); they have been widely used in ecotoxicological studies, permitting easy comparisons. They consist of 80% anatase and 20% rutile with a nominal diameter of 25.0 ± 5.7 nm; are polyhedral, uncoated, with a specific surface area of 46 ± 1 m² g⁻¹ and zeta potential in ultrapure water of -10.5 ± 2.6 mV (File S1).²² The TiO₂ NP suspension was freshly prepared before soil contamination (10000 mg L⁻¹); it was vortexed for 30 s and agitated while pipetting to keep it homogenized.

1.2. Terrestrial exposure

1.2.1. Exposure medium preparation

The two main conditions in this study were the control soil and the spiked soil, from which all conditions were derived. Soils were silty sand (Lufa Speyer 2.1; File S2) prepared with 20% (w/w) commercial compost to sustain proper plant growth (final soil mixture pH: 5.3, organic matter content: 12.6%). For the spiked soil, 500 mg kg⁻¹ of TiO₂ NPs were added to mimic several years of sewage sludge spreading on agricultural soils.¹⁵ Both control and spiked soils were mixed with either Millipore water or with NP suspension at a ratio of 1: 1 (w:w). After shaking at 100 rpm for 2 h, soils were filtered to eliminate excess water, dried, and transferred to

small plastic pots, $7 \times 7 \times 6.4$ cm (Soparco, France). This mixing procedure was chosen to ensure a homogenous contamination of the soil matrix.

1.2.2. *Medicago truncatula* exposure to TiO₂ NPs

Medicago truncatula (ecotype A17) is a model for legume plants,²³ chosen for their implication in the nitrogen cycle.²⁴ Seeds were kindly provided by Dr. Manuel Gonzalez-Guerrero, Universidad Politécnica de Madrid, Spain. They were first scarified and soaked overnight in water, in the dark. They were then placed in Petri dishes (6 to 7 seeds/Petri dish) with Hoagland agar medium for germination at 4°C for 72 h, before transferring to the soil pots. Seedlings ($n = 10$ per condition) were biomonitored every day for the number of leaves, height, and chlorophyll levels using a SPAD-meter (SPAD-502 chlorophyll meter, Minolta Camera Co., Japan). After 60 days, plants were harvested. Dry weights were recorded after 48 h at 60°C prior to Ti quantification in leaves.

1.2.3. Impact of TiO₂ NPs on soil microbial communities

The microbiological analysis focused on soil nitrifiers and denitrifiers to further investigate NP impact on the nitrogen cycle. Upon plant harvest, three cores of whole-depth bulk soil, deprived of roots (and without further processing), were collected at 3 cm from the stem axis from individual soil pots ($n = 6$ per condition). The three cores were homogenized, and 300 mg composite subsamples were stored at -20°C until DNA isolation. The FastDNA SPIN Kit for Soil was used according to the manufacturer's instructions (MP Biomedicals laboratories, Unites States). The quality and quantity of DNA extracts were determined using a NanoDrop spectrophotometer. Quantitative PCR (qPCR) was used to measure the abundance of total soil

bacteria targeting the universal 16S rRNA gene as well as archaeal ammonia monooxygenase A genes (*amoA*) for ammonia oxidizing archaea (AOA, nitrifiers), bacterial *amoA* for ammonia oxidizing bacteria (AOB, nitrifiers), and the bacterial nitrite reductase (*nirK* and *nirS*) and the nitrous oxide reductase (*nosZ*) genes for denitrifiers.²⁵ For more details about the experimental procedure, see File S3.

1.2.4. *Helix aspersa* exposure to TiO₂ NPs

Snails were chosen for this experiment as sensitive bioindicators of pollution in the environment.²⁶ They were obtained from a snail breeding farm to ensure homogeneity. After a 48 h depuration step, they were transferred to clean boxes containing 5 g of dry soil (after plant harvest) mixed with 3 mL of Millipore water ($n = 6$ per condition). Soil moisture was set high (189% of the water holding capacity), to sustain high hygrometry in microcosms and thus snail activity. The experimental model included only soil exposure, which was renewed each day for 10 days; snail feces were collected daily. After removal, soils were dried and weighted to determine the quantity consumed by each snail over 24 h. Following 72 h of depuration, snails were rinsed and stored at -20°C . Snail foot and viscera were separated and dried prior to Ti quantification.²⁶

1.3. Aquatic exposure

1.3.1. Exposure media for amphibian larvae

Exposure media for amphibian larvae were prepared using a new batch of control and spiked soils. Soils (1 kg of dry matter) were mixed with 10 volumes (ratio 1:10) of mineral water (Volvic®; Ti under detection limit of inductively coupled plasma–optical emission spectroscopy

[ICP-OES]) according to the adapted static leaching procedure of the French norm XP 31-210 (AFNOR, 1992) without filtration.²⁹ After 24 h of stirring, the mixtures were allowed to settle for 2 h, and the supernatant suspensions were collected by siphoning into glass flasks. Ten different exposure conditions were set up (Table 1).

Table 1: Amphibian larvae exposure conditions (CS : control soil, SS : spiked soil)

Condition	Preparation
NC	Pure Volvic® water
PC	40 mg L ⁻¹ cyclophosphamide in Volvic® water
ICP equiv of CS	3.6 mg L ⁻¹ TiO ₂ NPs in Volvic® water
CS Raw	Raw leachates from control soil
CS50	50% raw leachates from control soil + 50% Volvic® water
CS25	25% raw leachates from control soil + 75% Volvic® water
ICP equiv SS	12.3 mg L ⁻¹ TiO ₂ NPs in Volvic® water
SS Raw	Raw leachates from spiked soil
SS50	50% raw leachates from spiked soil + 50% Volvic® water
SS25	25% raw leachates from spiked soil + 75% Volvic® water
NC = negative control; PC = positive control (genotoxicity assay); ICP equiv of CS = equivalent Ti concentration of CS in Volvic®; ICP equiv SS = ICP equivalent Ti concentration of SS in Volvic®.	

1.3.2. Impact of TiO₂ NPs on *Xenopus laevis*

X. laevis, a widely used model in ecotoxicology,^{27,28} are filter-feeders, and can be contaminated via skin contact, water filtration *via* gills, and breeding exposure. *X. laevis* larvae (stage 50 of the development table of Nieuwkoop & Faber³⁰) were exposed in semi-static conditions based on modified ISO 21427-1.³¹ The larvae ($n = 15$ per condition) were taken from the same hatch to reduce inter-animal variability. They were submitted to daily renewal of media and fed with fish food TetraPhyll®. After 12 days of exposure, mortality was recorded and larvae anaesthetized (MS222, Sandoz, France) to take pictures and evaluate growth inhibition (Image J). Genotoxicity was assessed through the micronucleus test³¹: blood was extracted from the heart of each anesthetized larva, and micronucleated erythrocytes per thousand cells (ME‰) were counted under optical microscope following hematoxylin-eosin staining.

1.4. Ti uptake: acid digestion and ICP-OES

Acid digestion was done in a DigiPrep® heating system, using HNO₃, NH₄F, and HClO₄ as well as H₂O₂, with a temperature of 100°C and several evaporation steps.¹¹ Digested samples were diluted in 5% HNO₃. For soil, a filtration step was necessary (0.45 µm syringe filters). The samples were then analyzed by ICP-OES with control samples (blanks with only chemicals and standard reference material NIST 1573a: tomato leaves) (IRIS Intrepid II XDL, Thermo Scientific, United States). Ti recovery from TiO₂ NPs has been calculated to be $88.2 \pm 2.1\%$.¹¹

1.5. Statistical Analysis

Data were checked for homoscedacity and normality. When assumptions were met for parametric analyses, a Welch *t*-test or a one way ANOVA were used. Otherwise, a Wilcoxon test

or a Kruskal-Wallis test followed by a Dunn's post hoc test were applied. A principal component analysis (PCA) was carried out on plant elemental content to investigate TiO₂ NP impact on plant nutrition. All statistical analyses were performed using the R statistical software (version 3.1.3),³² with *car*,³³ *multcompView*,³⁴ *pgirmess*,³⁵ and *lsmeans*³⁶ packages (all results are gathered in Table S4). Data is displayed as median \pm interquartile range, average is shown by a red dot.

2. RESULTS AND DISCUSSION

2.1. Terrestrial ecosystem

2.1.1. Ti concentration in soil

As expected, the addition of TiO₂ NPs lead to a significant increase in Ti concentration in spiked soil in comparison with the control soil ($p = 0.01$). The average concentration in the control soil was $278.0 \pm 13.4 \text{ mg kg}^{-1}$, while that in spiked soil was $341.7 \pm 12.2 \text{ mg kg}^{-1}$ (see File S5A). Although the soils were spiked with 500 mg kg^{-1} TiO₂ NPs ($300 \text{ mg Ti kg}^{-1}$ equivalent), the average increased concentration of Ti in spiked soils after leaching was below 300 mg kg^{-1} Ti. Considering that the soil used in this study was 88% sandy, this could be explained as a loss of Ti into the water compartment during the leaching procedure, as suggested by our previous results.¹¹

2.1.2. TiO₂ NP phytotoxicity and uptake by *Medicago truncatula*

After 60 days of exposure, plants grown in the spiked soil were significantly smaller than those grown in the control soil ($p < 0.001$) with average heights of $4.3 \pm 0.3 \text{ cm}$ and $6.2 \pm 0.3 \text{ cm}$,

190 respectively (Figure 1A). Plant heights were similar until day 12, after which exposed plants had
191 a 17% smaller size (File S5B). Along the development, non-exposed plants produced
192 significantly more leaves ($p = 0.01$) with *ca.* seven against five leaves for exposed plants (Figure
193 1B). A significant reduction in aboveground biomass (-53%) was also observed for exposed
194 compared to non-exposed plants ($p < 0.001$; File S5C). Chlorophyll levels tended to be lower in
195 the spiked conditions than in the control conditions, but no significant difference was detected
196 (File S5D). In terms of Ti uptake, concentration in the exposed leaves was significantly higher
197 compared to the non-exposed leaves ($p < 0.01$) with respective concentrations of 196.6 ± 53.1
198 mg kg^{-1} and $37.6 \pm 7.9 \text{ mg kg}^{-1}$ (Figure 1C). Exposure to TiO_2 NPs tended to impact plant
199 ionome: exposed plants were significantly enriched in zinc, lead, sodium, manganese,
200 phosphorus but contained less copper, iron, potassium, sulfur, molybdenum, and boron (Figure
201 1D).

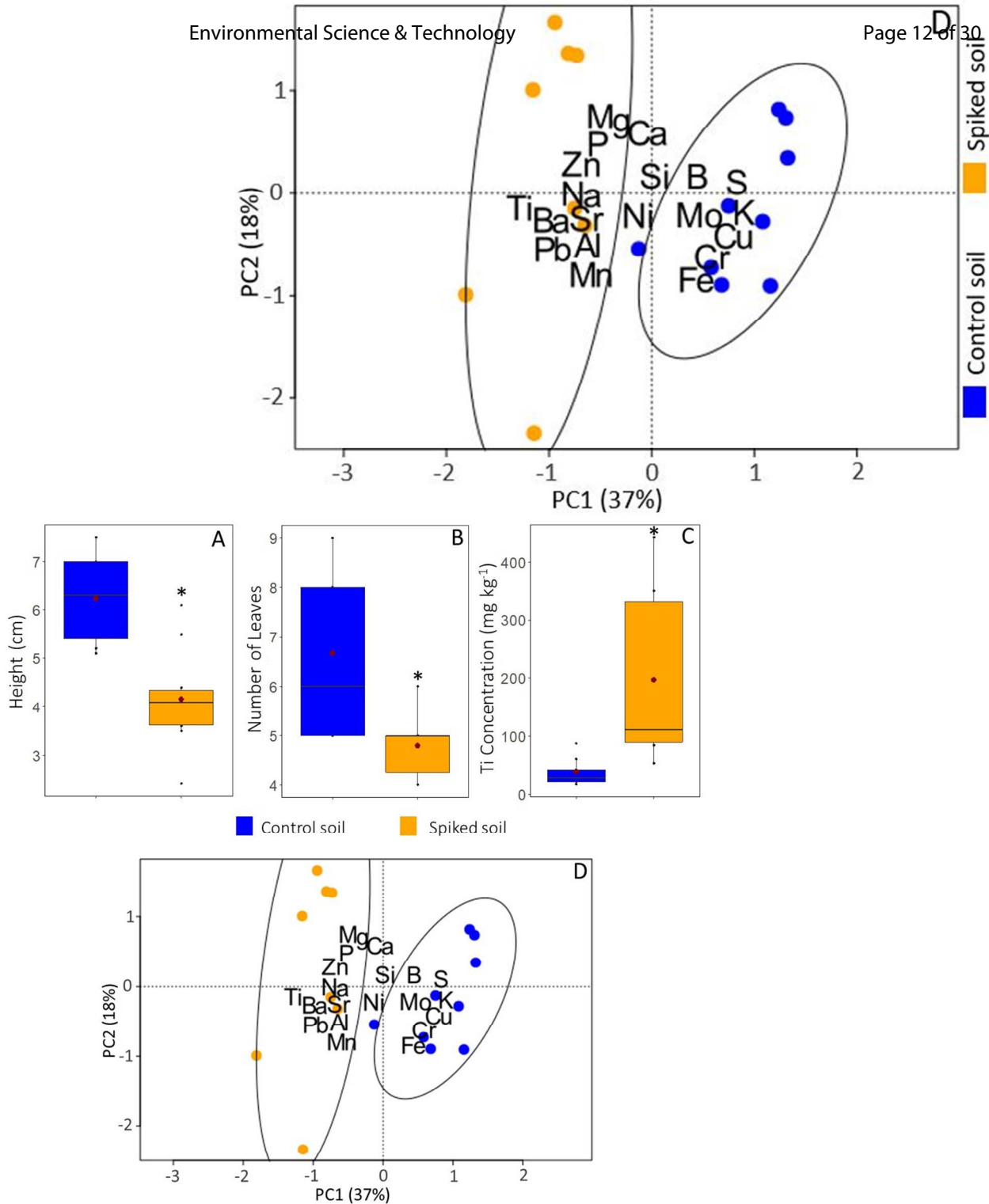


Figure 1: A: Impact of TiO_2 NPs on plant (*M. truncatula*) growth; B: Impact of TiO_2 NPs on the number of plant leaves; C: Ti concentrations measured by ICP-OES in the aboveground biomass of plants; D: Principal component analysis indicating the impact of TiO_2 NPs on the elemental content in the leaves. Asterisks represent significant differences ($p < 0.05$). Average Ti concentrations in spiked soil = $341.7 \pm 12.2 \text{ mg kg}^{-1}$; average Ti concentrations in control soil = $278.0 \pm 13.4 \text{ mg kg}^{-1}$. Exposure: 60 days; $n = 10$.

210 The significant differences between non-exposed and exposed plants indicate an impact of TiO₂
211 NPs tending to inhibit plant growth and shoot biomass. Rafique *et al.*³⁷ showed that TiO₂ NP
212 concentrations of 20 to 60 mg kg⁻¹ (sandy loam) led to a dose-related increase in root and shoot
213 length and biomass in wheat plants exposed for 60 days. However, they demonstrated that
214 concentrations of 80 to 100 mg kg⁻¹ gradually inhibited root and shoot length and reduced plant
215 biomass. In our study, Ti concentrations were fivefold higher in the plants exposed (341.7 mg kg⁻¹)
216 for 60 days when compared with the non-exposed plants. Our results also conform to those
217 obtained in a recent study by Larue *et al.*, which showed that wheat plants exposed for 3 weeks
218 to sandy soils spiked with 500 mg kg⁻¹ of TiO₂ NPs took up significantly more Ti than the
219 control.¹¹ In addition, plants initially developing at the same rate began to show a consistent
220 change in growth rate by the beginning of the third week of exposure. We hypothesize that one
221 reason for the significant difference in plant development is the impact of TiO₂ NPs on plant
222 ionome. Among the deficient elements in plants grown in TiO₂ NP-spiked soils is potassium,
223 which is essential for plant growth, development, regulation, and crop yield.³⁸ Maathuis³⁹ posited
224 that at low potassium levels, sodium can replace potassium due to its structural similarity, which
225 is evidenced in our study, where the plants deficient in potassium are enriched, instead, in
226 sodium. Pi *et al.*⁴⁰ showed that when plants were exposed to stress (K deficient environment),
227 they replaced K⁺ with Na⁺, but did not recover to the same extent as the control group, which
228 could explain why the exposed plants were significantly smaller and less productive—with a
229 deficiency in potassium—than the non-exposed plants. However, contrary to our results, Servin
230 *et al.*⁴¹ demonstrated an increase in potassium in cucumber plants exposed to TiO₂ NPs. This
231 difference might be attributed to the plant models used. Also in line with our results, Rafique *et*
232 *al.*⁴² showed an increase in phosphorus availability in wheat plants exposed to TiO₂ NPs. In

addition, ICP analysis showed that spiked soil plants were deficient in other micronutrients, such as boron, copper, iron, and molybdenum, which are necessary for the plant physiological processes⁴³ and, therefore, could be explanative of the significant reduced development of the exposed plants.

2.1.3. Impact of TiO₂ NPs on soil microbial communities

There was no difference between the abundance of the total bacteria (16S rRNA) in the control and spiked soils ($p = 0.74$), with the average number of gene copies per gram of soil being $1.92 \times 10^{10} \pm 1.95 \times 10^9$ and $1.83 \times 10^{10} \pm 1.97 \times 10^9$, respectively (Figure 2).

Evaluation of *amoA* genes, both for AOA and AOB, indicated a significant decrease in abundance in the spiked soil ($p = 0.007$ and $p = 0.001$, respectively). The average number of gene copies/g soil was $2.97 \times 10^7 \pm 2.48 \times 10^6$ for non-exposed AOA and $2.07 \times 10^7 \pm 1.31 \times 10^6$ for exposed AOA, while those for non-exposed AOB and exposed AOB were $1.90 \times 10^7 \pm 1.04 \times 10^6$ and $1.05 \times 10^7 \pm 1.11 \times 10^6$, respectively (Figure 2).

In the case of the denitrifying genes *nirK*, *nirS*, and *nosZ*, no significant difference was found between the control and spiked soils ($p = 0.49$, $p = 0.76$, and $p = 0.67$, respectively). The average number of gene copies/g soil obtained for *nirK*, *nirS*, and *nosZ* were $3.81 \times 10^8 \pm 3.47 \times 10^7$, $2.00 \times 10^7 \pm 1.80 \times 10^6$, and $1.91 \times 10^9 \pm 1.52 \times 10^8$, respectively (Figure 2).

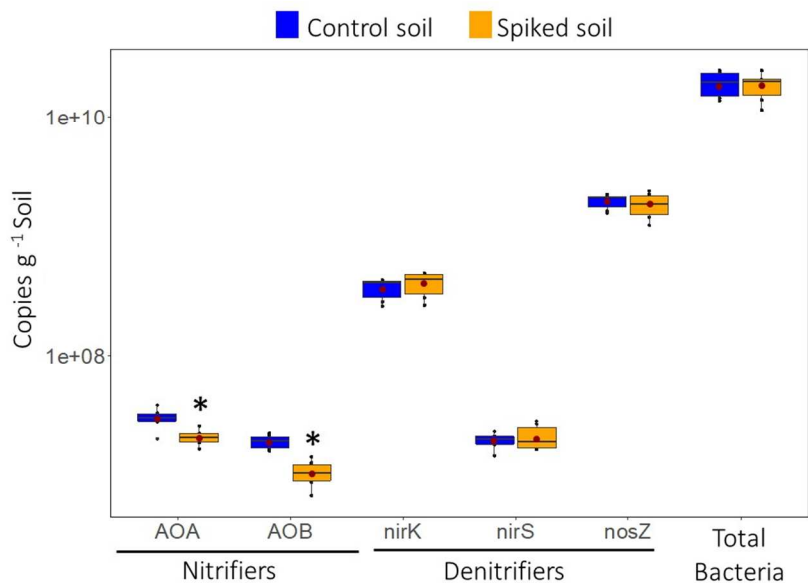


Figure 2: Impact on bacterial nitrifier and denitrifier genes. Nitrifier genes include the ammonia oxidizing archaea (AOA) and the ammonia oxidizing bacteria (AOB) ammonia monooxygenase A gene (*amoA*). The denitrifier genes include the bacterial nitrite reductase genes (*nirK* and *nirS*) and the bacterial nitrous oxide reductase gene (*nosZ*) for denitrification. Values on the y axis are expressed on the logarithm scale. Asterisks represent significant differences ($p < 0.05$). Average Ti concentrations in spiked soil = $341.7 \pm 12.2 \text{ mg kg}^{-1}$; average Ti concentrations in control soil = $278.0 \pm 13.4 \text{ mg kg}^{-1}$. Exposure: 60 days; $n = 6$.

The literature concerning the impact of TiO_2 NPs on the soil microbial communities is scarce. Ge *et al.*^{44,45} and Simonin *et al.*⁴⁶ showed reductions in bacterial biomass and shifts in the community structure at high to very high TiO_2 NP concentrations (up to 20 g kg^{-1}). In our experiment, the total bacterial abundance was not affected probably because the TiO_2 NP concentration was lower. However, the relative abundance of some taxa decreased (soil nitrifiers vs. denitrifiers), suggesting potential consequences on bacterial community functionalities. Interestingly, Simonin *et al.* evidenced a lack of the classical dose–response relationship.⁴⁶ This phenomenon was explained by the concentration-dependent modifications of NP properties involved in their bioavailability and ecotoxicity.²⁵ Simonin *et al.*⁴⁶ showed a 40% reduction in AOA abundance at TiO_2 NP concentrations from 0.05 to 500 mg kg^{-1} . The magnitude of AOA decrease in our experiment was $35.9 \pm 4.5\%$, which is in agreement. In the case of AOB,

Simonin *et al.*⁴⁶ evidenced an unusual “U” shape for dose response, with intermediate concentrations of TiO₂ NPs significantly decreasing the AOB abundance, and the lowest and highest concentrations showing no significant decrease. Based on the model derived from their experimental data ($y = 0.009 \times \ln ([\text{TiO}_2])^2 - 0.0215 \times \ln ([\text{TiO}_2]) - 0.26$), the estimation of AOB decrease at 341 mg kg⁻¹ would be 6.5%. In our study, we measured a higher decrease: 44.9% ± 7.3%. While Simonin *et al.*⁴⁶ attributed the unusual dose-response relationships to the modification of TiO₂ NP properties based on their concentrations (e.g., aggregation of TiO₂ NPs at intermediate and higher concentrations), the discrepancy between results for AOB obtained in their study and ours might be explained by the difference between soil types and thereby between soil–NP interactions. Simonin *et al.*⁴⁶ used silty clay, whereas our study used silty sand, highlighting the importance to consider soil type when studying NP fate in the environment. Bissett *et al.*⁴⁷ discussed the relative insensitivity of denitrifiers to environmental toxicants, and attributed the resistance of this functional guild to their high functional redundancy, niche breadth, and adaptive ability compared with the soil nitrifiers, which have limited phylogenetic diversity. In terms of relationship between plants and soil bacteria, we hypothesize that another reason for the inhibition in plant development, and the delay of this impact until the third week, could be an indirect effect of TiO₂ NPs on the soil nitrifier community. Indeed, Van Der Heijden⁴⁸ discussed how soil bacteria indirectly affect plant productivity by altering nutrition and resource availability.

2.1.4. Toxicity and uptake of Ti by *H. aspersa*

No difference in snail mortality was detected along the exposure period. The analysis of total soil consumption by snails from day 1 to day 10 did not reveal any significant difference between

treatments ($p = 0.56$): the average daily soil consumed by snails amounting to 1.36 ± 0.17 g for spiked soil and to 1.23 ± 0.01 g for control soil (data not shown). Ti concentration in snail foot, viscera, and feces did not show significant difference ($p = 0.78, 0.49, 0.88$, respectively). Ti uptake in the non-exposed snail foot was 0.33 ± 0.03 mg kg⁻¹ and in the exposed snail foot was 0.86 ± 0.30 mg kg⁻¹ (Figure 3A), while for the viscera, the average Ti concentrations after depuration were 0.39 ± 0.12 mg kg⁻¹ and 0.28 ± 0.08 mg kg⁻¹ for non-exposed and exposed snails, respectively (Figure 3A). Average Ti concentrations in feces were 367.78 ± 52.41 mg kg⁻¹ for non-exposed snails and 377.51 ± 38.88 mg kg⁻¹ for exposed snails (Figure 3B).

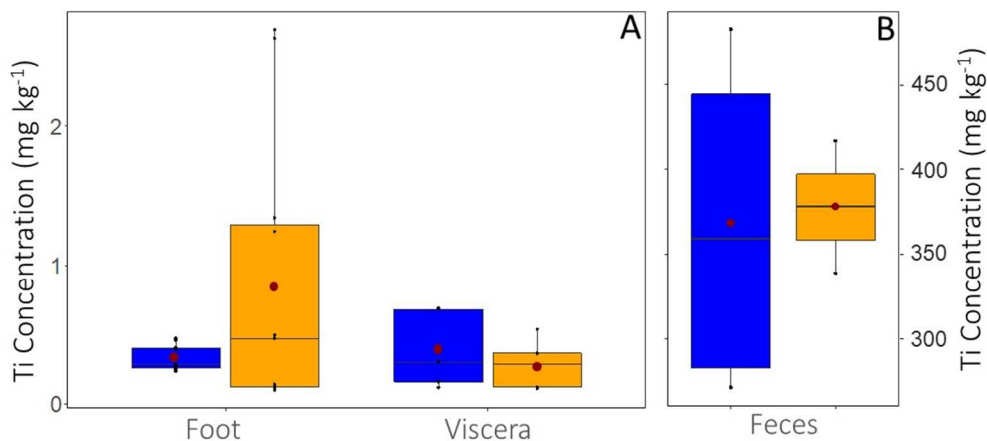


Figure 3: A: Ti uptake in snail (*H. aspersa*) foot and viscera; B: Ti concentration in snail feces, measured by ICP-OES. Average Ti concentrations in spiked soil = 341.7 ± 12.2 mg kg⁻¹; average Ti concentrations in control soil = 278.0 ± 13.4 mg kg⁻¹. Snails were exposed to 5 g each of control or spiked soils every day. Exposure: 10 days; $n = 6$.

The total soil consumed by snails in the two treatments was not significantly different, which highlights that there was no behavior of avoidance of the contaminated soil. There was also no significant difference in sequestration of Ti from soil into snail foot and viscera, or in the excretion in feces. Concentrations in the snail feces show that Ti was quickly eliminated from the snail body via feces. The higher concentrations of Ti in the feces of both the control and spiked

conditions compared to the soil concentrations is presumably due to the digestion of soil organic matter, and thus to a concentration of its mineral fraction. While results on the effects on land snails are in lack, Yeo and Nam¹⁹ performed a paddy microcosm study, in which TiO₂ NPs were found to transfer from prey to consumer (biofilm/water dropwort to nematodes/snail) within a period of 17 days, highlighting the potential for bioaccumulation in the food chain. In 2006, Scheifler *et al.*⁴⁹ evaluated the transfer of several heavy metals in a soil–plant–invertebrate microcosm, using lettuce as primary producer and the snail *H. aspersa* as primary consumer. They demonstrated that while copper and nickel were not accumulated in the snail body, there was a gradual increase in cadmium and zinc, and a rapid accumulation of lead in snails, but more than 80% of this accumulation was contributed from soil exposure rather than plant exposure. Similarly, other studies have demonstrated significant increases in concentrations of metals such as chromium and platinum from contaminated food in *H. aspersa* after 56 days⁵⁰. Although our results did not show any significant uptake in snail, there was a trend for higher Ti concentrations in snail foot under NP exposure, *ca.* two-fold higher on average than in controls. The absence of a significant effect may be due to the high variability in the concentrations measured, reducing statistical power. Including more replicates and a longer exposure period may be needed to reach a conclusion.

2.2. Aquatic ecosystem

2.2.1. Ti in amphibian exposure medium

Titanium in the amphibian exposure media showed a higher concentration of $12.3 \pm 0.15 \text{ mg L}^{-1}$ in the raw spiked soil leachates compared to $3.6 \pm 0.18 \text{ mg L}^{-1}$ in the raw control soil leachates (*p*

= 0.01). Whereas the ratio of Ti found in control soil versus spiked soil was 1:1.3, the ratio of Ti found in control soil leachates versus spiked soil leachates was 1:3.4.

In the present study, a real environmental exposure model in the aquatic system was designed, taking into account an approximation of expected percentage of TiO₂ NP concentrations coming from the leaching of an agricultural soil into surface waters. Leachate Ti concentrations were much higher than the predicted concentrations of 2.17 µg L⁻¹ TiO₂ NPs in surface waters by Sun *et al.* (2016), which is attributed to the high concentration of soil spiking in our study. The increased ratios of Ti in the spiked soil leachates compared with the control soil leachates mean that more Ti from the spiked soil moved into the water compartment during the preparation of soil leachates than from the control soil. Gogos *et al.*⁵¹ showed a significantly lower transfer of Ti from contaminated soil to soil leachate (0.001% vs. 26% in our study) but with a different leaching procedure (filtration of the NP suspension through the soil layer vs. run-off simulation over a soil including soil particles with no filtering step).

2.2.2. Impact of TiO₂ NPs on *Xenopus laevis* larvae

There was no significant mortality (<20%) among amphibian larvae exposed to TiO₂ NPs. A comparison of the larval lengths after 12 days of exposure showed a significant difference among groups ($p < 0.0001$) (Figure 4A). There was no significant decrease in the length of the larvae exposed to the ICP equivalents of both the spiked and control soil leachates compared to the negative control larvae (4.77 ± 0.09 cm and 4.77 ± 0.14 cm, respectively, vs. 4.46 ± 0.09 cm). Larvae exposed to raw spiked and control soil leachates (3.31 ± 0.14 cm and 3.17 ± 0.17 cm, respectively) and 50% diluted spiked and control leachates (3.21 ± 0.14 cm and 3.30 ± 0.13 cm,

360 respectively) showed significant lower size compared to the negative control larvae and to the
361 both ICP equivalents. Larvae exposed to 25% diluted spiked and control soil leachates also
362 showed a significantly reduced size (3.54 ± 0.14 cm and 3.70 ± 0.11 cm, respectively) compared
363 to both the ICP equivalents, but no significant lower length compared to the negative control
364 larvae. In addition, the larval size tended to increase with the leachate dilution. There was a
365 significant genotoxicity ($p < 0.0001$ among groups) in larvae exposed to the ICP equivalent of
366 the spiked soil leachate (6.60 ± 0.97 micronuclei), to the spiked and control soil raw leachates
367 (13.15 ± 1.81 and 6.08 ± 0.49 micronuclei, respectively), and to the 50% diluted spiked soil
368 leachate (6.07 ± 0.90 micronuclei), compared to the negative control larvae (1.07 ± 0.30
369 micronucleus) (Figure 4B). There was no significant genotoxicity in larvae exposed to the ICP
370 equivalent of the control soil leachate (4.73 ± 1.09 micronuclei), to the 50% diluted control soil
371 leachate (3.42 ± 0.82 micronuclei), and to the 25% diluted spiked and control soil leachates (2.57
372 ± 1.11 and 3.50 ± 0.81 micronuclei, respectively) compared to the negative control larvae.

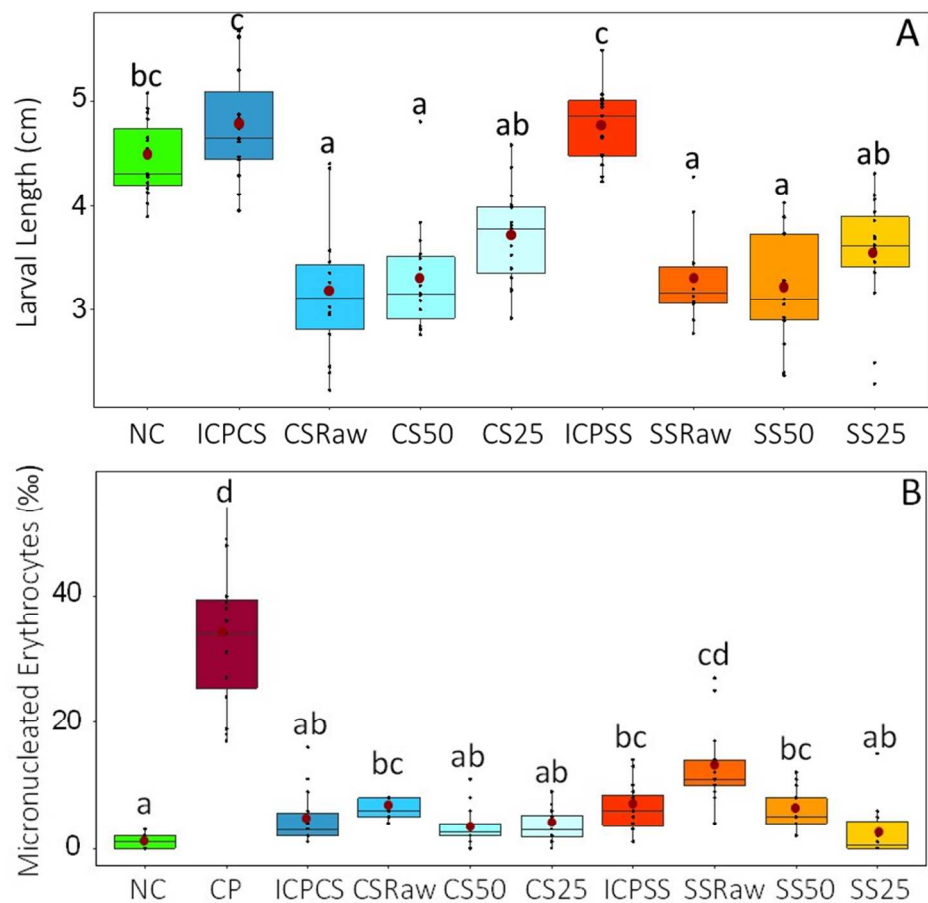


Figure 4: A: Impact of Ti on amphibian (*X. laevis*) larval length; B: Percentage of micronucleated erythrocytes (ME%). Average Ti concentration in the control soil leachates was 3.6 mg L⁻¹ and that in the spiked soil leachates was 12.6 mg L⁻¹. Exposure: 12 days; *n* = 15. NC = negative control; PC = positive control; ICP CS = ICP equivalent of control soil leachates (3.6 mg L⁻¹ TiO₂ NPs in Volvic®); CS Raw = raw control soil leachates; CS 50% = 50% diluted control soil leachates in Volvic®; CS 25% = 25% diluted control soil leachates in Volvic®; ICP SS = ICP equivalent of spiked soil leachates (12.6 mg L⁻¹ TiO₂ NPs in Volvic®); SS Raw = raw spiked soil leachates; SS50% = 50% diluted spiked soil leachates in Volvic®; SS 25% = 25% diluted spiked soil leachates in Volvic® water. Alphabets represent statistically different groups (*p* < 0.05).

Our results demonstrated the inhibition potential of soil leachates on larval growth, and a genotoxicity of both soil leachates and TiO₂ NPs. Mouchet *et al.*²⁹ already demonstrated a toxicity of leachates from contaminated soil to amphibian larvae. Although Chenon *et al.*⁵² showed that sludge-amended soil leachates, including non-lethal dilutions, lead to a decrease in size of amphibian larvae, in our study, the impact of raw control soil leachates was an

unexpected finding. Therefore, we performed an ICP-OES analysis separately on the compost for elemental concentrations. The results showed an average lead concentration of 9.8 mg kg^{-1} , which might have resulted in a cocktail effect with TiO_2 NPs, as Mouchet *et al.*⁵³ found lead concentrations from 1 mg L^{-1} to be genotoxic to amphibian larvae. In addition, according to Bekaert *et al.*,⁵⁴ pollutants adsorbed onto soil particles may be biologically active when compared with those on filtrates, and as a result, in our study, the presence of soil particles may have contributed to the impact on amphibian growth. The genotoxic potential of TiO_2 NPs was evidenced by the increase in the number of micronucleated erythrocytes in larvae exposed to the ICP equivalent of/and the raw spiked soil leachates. Bacchetta *et al.*⁵⁵ also demonstrated the toxicity of TiO_2 NPs in *X. laevis* when exposed for 96 h to concentrations ranging from 10 to 500 mg L^{-1} , resulting in deformities in embryo development. Previous studies have already shown the negative effects of TiO_2 NPs on aquatic organisms such as *X. laevis*⁵⁶ and *Danio rerio*,⁵⁷ causing deformities and death, while Federici *et al.*⁵⁸ showed that TiO_2 NPs had toxic effects in rainbow trout at concentrations $<1.0 \text{ mg L}^{-1}$, which could indicate a difference in sensitivities among species. The ICP equivalents of both the raw control soil and raw spiked soil leachates had half or less than half the micronucleated erythrocytes compared with both the raw leachates, respectively, which, again, could be due to a cocktail effect resulting from the presence of lead in the compost.

Our study highlights the potential risks of TiO_2 NPs in the environment, showing an upward transfer of Ti in the terrestrial ecosystem, from soil to plants, and a downward transfer of Ti from soil to the amphibian exposure medium. The inhibition of nitrifiers due to the presence of TiO_2 NPs can have far-reaching consequences, especially for soil fertility, global ecosystem functions/services, and crop production. The indirect effect of nitrifiers on crop production, and

the uptake of Ti by alfalfa plants, lead to the question of food security. Although we did not find significant uptake of Ti by snails, the concentration-dependent sequestration of Ti in snail foot deserves some attention with the possibility for transfer up the food chain and bioaccumulation over the years. Finally, runoff from contaminated soils ends up in freshwater ecosystems and can be genotoxic to amphibian larvae. By focusing on several experimental models rather than a single-species study, we provide data concerning the distribution of TiO₂ NPs between the terrestrial and aquatic ecosystems, the levels of the terrestrial ecosystem that are most affected—soil bacteria and primary producers—and the risk of toxicity in aquatic ecosystems by leaching of Ti from soil. Three-fourths of our experimental models have been affected by TiO₂ NPs, which is sufficient to acknowledge that precautionary standards for TiO₂ NPs are required. These ecotoxicological effects can be used in future studies to identify safe threshold values of TiO₂ NPs in the environment and suitable waste disposal methods to ensure that NPs do not reach such high concentrations as identified to be toxic in our study.

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Supporting Information Available

This information is available free of charge via the Internet at <http://pubs.acs.org>.

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