

Communication

Distribution of TiO₂ Nanoparticles in Acidic and Alkaline Soil and Their Accumulation by Aspergillus niger

Martin Šebesta^{1,*}, Lucia Nemček¹, Martin Urík¹, Marek Kolenčík^{2,3}, Marek Bujdoš¹, Ingrid Hagarová¹ and Peter Matúš¹

- ¹ Institute of Laboratory Research on Geomaterials, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovičova 6, 842 15 Bratislava, Slovakia; lucia.nemcek@uniba.sk (L.N.); martin.urik@uniba.sk (M.U.); marek.bujdos@uniba.sk (M.B.); ingrid.hagarova@uniba.sk (I.H.); peter.matus@uniba.sk (P.M.)
- ² Department of Soil Science and Geology, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Trieda A. Hlinku 2, 949 76 Nitra, Slovakia; marek.kolencik@uniag.sk
- ³ Nanotechnology Centre, VŠB Technical University of Ostrava, 17. listopadu 15/2172, 708 33 Ostrava, Czech Republic
- * Correspondence: martin.sebesta@uniba.sk; Tel.: +421-2-602-96-289

Received: 29 September 2020; Accepted: 19 November 2020; Published: 22 November 2020



Abstract: The nanoparticles of TiO_2 (TiO_2 NP) have been used as a plant-growth stimulant or catalyst in pesticide formulas. However, due to high resistance of TiO₂ NP to abiotic weathering, dissolved Ti is unlikely to act as an active compound in these preparations. Even if soil is acidic, TiO₂ NP do not dissolve easily and preferably remain as undissolved particles. The low dissolution rates of inorganic nanoparticles in the soil environment make Ti in TiO₂ NP largely unavailable for plants and soil microorganisms. To characterize the behavior of TiO_2 NP in soil under different pH conditions, we analyzed TiO₂ NP-size distribution in two soil materials, an alkaline and acidic one. We also cultivated Aspergillus niger, a fungus ubiquitously found in soils, in the growth medium spiked with TiO₂ NP to assess accumulation of the nanoparticles in fungus. In soil suspensions, the dissolved Ti was present in low concentrations (up to 0.010 mg L^{-1}). Most of the TiO₂ NP remained in particulate form or appeared as aggregates sized 100-450 nm. In experiment on Ti accumulation by A. niger, TiO₂ NP either settled down to the bottom of the flask with growth medium or were actually accumulated by the fungus; about 7.5% of TiO₂ NP were accumulated in fungal mycelia. Most of the TiO₂ NP remain in particulate form in soil solutions, regardless of soil pH. Filamentous fungus A. niger has the ability to accumulate bioavailable TiO2 NP, which hints at the possibility that some soil fungi can affect spatial distribution of this type of nanoparticles in soils.

Keywords: metal oxide nanoparticles; Ti mobility; soil; dissolution; transformation; bioavailability

1. Introduction

Titanium (Ti) is one of the most abundant elements in soil, sediments and weathered rocks. In natural waters, however, the levels of soluble Ti range from 4 pM at the surface of ocean waters [1] to 100 μ M in hot spring waters [2]. This is due to a very low dissolution rate and high resistance of Ti minerals to chemical weathering [3]. Its essentiality is also disputed [4,5]. It has just recently been found that Ti has a role in the growth and development of plants [4] and it acts as a plant-growth stimulant when in nanoparticulate form (TiO₂ NP) [6–12]. The TiO₂ NP are used as additives in pesticides and other complex formulations as either an active ingredient or a carrier. They increase adhesion or accelerate the degradation of the organic pesticides [13–17]. Yet, they can negatively affect roots and



symbiotic mycorrhizae of plants and fungi [18]. The recommended dosage varies with application and plant species. Generally, a single application of TiO_2 NP that does not increase nanoparticle concentration above the value of approx. 60 mg kg⁻¹ is considered to be safe for use [19,20].

The stability of nanoparticles in soils is strongly affected by pH. Their dissolution rate mostly increases with increasing soil acidity [21,22]. During the process of breathing, plants release CO₂ into the soil where it dissolves and forms carbonic acid. That acid reduces the pH of soil in the root area [23]. Plants also release organic acids into the environment to better mineralize and solubilize poorly available elements [24]. Soil microorganisms also partially contribute to this process [25] via complexolysis by excreting extracellular biogenic chelating metabolites. Moreover, a larger area of nanoparticles' surfaces is exposed to chemical weathering, which increases the dissolution rate. Therefore, non-aggregated smaller particles are affected by soil pH and microorganisms instantly and are more prone to dissolution [21,26,27]. Thus, TiO₂ NP may be among those Ti forms which dissolve first after being exposed to the weathering effects of acidic soil conditions, plant and microbial activity. During weathering, a significant mobilization of Ti in the soil due to acidification by inorganic and organic acids may occur [5,28].

Soil properties affect nanoparticle solubility, dispersion and aggregation, with soil pH being the dominant factor. Therefore we have turned our attention towards particle size distribution and solubility of TiO₂ NP in acidic and alkaline soil. The selected particle size ranges were 1–100, 100–450 and 450–1000 nm. The concentration of dissolved Ti was measured by centrifugal ultrafiltration. The distribution of TiO₂ NP in soils is not only affected by nanoparticle dissolution and aggregation but also by their accumulation in the tissues of soil organisms. The filamentous fungus *Aspergillus niger* has been found to be capable of producing a high amount of organic compounds that chelate metals and solubilize mineral phases. Furthermore, it can reduce pH levels in the surrounding soil environment [29–31] and thus has a greater potential to accumulate TiO₂ NP and Ti released from the nanoparticles. Moreover, *A. niger* has been observed to transform and mineralize biogenic TiO₂ NP [32–34]. For all these reasons, this particular species appeared to be a suitable one on which to experiment for the purpose of evaluation of fungal ability to accumulate TiO₂ NP from a liquid growth medium.

2. Materials and Methods

2.1. Soils Used in the Experiment

Samples of two soils differing in their characteristics were collected from 5 to 15 cm depth at two sites in the western part of Slovakia. The soils were classified according to the Morphogenetic Soil Classification System of Slovakia [35] and the World Reference Base for Soil Resources (WRB) [36] as Cernozem kultizemna karbonatova/Calcic Chernozem (CH-cc) and Kambizem luvizemna pseudoglejova/Dystric Stagnic Cambisol (CM-st.dy). CH-cc is an agricultural soil and was selected as a representative of soils with neutral to alkaline pH, while CM-st.dy represented natural forest soils with acidic pH. Digestion of 1 g of soil by the acid mixture of $HF + HNO_3 + HClO_4 + H_2O_2$ (4:3:4:1, 60 mL) in an open system at 200 °C was used to determine total concentrations of Al, Fe, Mn, Ti and Zn. Oxalate-extractable fractions of Al, Fe and Mn were determined using extraction with 0.2 mol L⁻¹ ammonium oxalate stabilized at pH 3 on samples ground to a particle size below 0.5 mm [37]. The method by Walkley and Black [38] was used to determine total organic carbon (TOC). Soil pH was determined in 1 mol L⁻¹ KCl (pH_{KCl}) and deionized water (pH_{H₂O) at a 1:2.5 soil to} solution ratio. The concentration of CaCO₃ was determined by gas volumetric measurement of CO₂ evolved upon 10% HCl treatment using calcimeter apparatus [39]. The size distribution of soil particles was determined by a pipette method [39]. The USDA-FAO soil-texture triangle was used to classify soil samples into textural classes [40]. Concentrations of humic substances, humic acids and fulvic acids in soil samples were determined by the method developed by Kononova and Belchikova [41]. The soil cation exchange capacity (CEC) was measured by the Kappen method [42]. The characteristics of the two soils are shown in Table S1 (Supplementary Material).

2.2. Batch Experiment with Soil

TiO₂ NP (nanopowder, 21 nm primary particle size (TEM), \geq 99.5% trace metals basis, Prod. No 718467, Sigma-Aldrich, Saint-Louis, MO, USA) used in the experiment were characterized with X-ray diffraction (XRD) (Bruker, MA, USA) and scanning electron microscopy (SEM) (JEOL JSM-7610F Plus, JEOL, Tokyo, Japan) and their detailed characteristics were reported in the previous work [11]. Results from SEM showed that TiO₂ NP were of nearly spherical shape with some nanocrystals being prismatic with bi-pyramidal termination. The XRD results revealed two distinct mineral phases, anatase and rutile, with their mean sizes calculated by XRD TOPAS 3.0 software (Burker, MA, USA) being 30.0 ± 2.0 and 19.6 ± 0.2 nm, respectively.

Suspension with the TiO₂ NP concentration of 100 mg L^{-1} was prepared by adding 0.50 mL of artificial rainwater concentrate (See Supplementary Material Section 2) and 50 mg of TiO₂ NP to a 500 mL volumetric flask that was then filled up to the mark with distilled water. The TiO₂ NP suspension was prepared shortly before the experiment and was placed in an ultrasonic bath for 15 min. This concentration was selected based on previous reports using a similar concentration in other experiments related to the transport of nanoparticles in soils [43].

Batch sorption experiments were conducted to explore the distribution of TiO_2 NP in liquidand solid-phase and to determine the amount of dissolved Ti in soil solutions. The 15 mL centrifuge tubes were filled with 0.790 mg of either CH-cc or CM-st.dy. Then, 15.8 mL of 100 mg L^{-1} TiO₂ NP suspension was added to the tubes. In a control sample, 15.8 mL of artificial rainwater was added. Each experimental set-up was done in triplicate. The sealed centrifugal tubes were mounted in an overhead shaker and shaken at 5 rpm for 24 h. After 24-h batch sorption, the centrifuge tubes were sequentially centrifuged at $1000 \times g$ for 1 min, $2300 \times g$ for 2 min and $3000 \times g$ for 30 min to separate particles or aggregates smaller than 1000 nm (C_{1000}), 450 nm (C_{450}) and 100 nm (C_{100}), respectively. Selected centrifugal forces and times were calculated according to the Stokes' law for particles made of TiO₂ [44–46]. After each step of centrifugation, 2 mL of supernatant was removed for the analyses of Ti content. After the last centrifugation step, 6 mL of supernatant was removed and added to ultrafiltration centrifugation units (Sartorius Vivaspin[®] 6 mL, 3 kDa), and the units were centrifuged for 20 min at $3500 \times g$. Then, 1.5 mL of filtrate was removed to analyze the sample for Ti content (C_1) . From the acquired Ti concentrations, the concentrations of Ti present in different particle size fractions were calculated: $450-1000 \text{ nm} = C_{1000}-C_{450}$; $100-450 \text{ nm} = C_{450}-C_{100}$; $1-100 \text{ nm} = C_{100}-C_1$; and dissolved Ti (C_1). The concentrations of Ti added in soil in the form of TiO₂ NP were calculated as a difference between Ti concentrations determined in the experiments with TiO₂ NP and concentrations of Ti naturally present on a site (control samples).

2.3. Cultivation of Fungus Treated with TiO₂ Nanoparticles

For the experiment on a microscopic filamentous fungus *Aspergillus niger* (Tiegh.) supplemented with different concentrations of TiO₂ NP, the fungus was grown in Sabouraud growth medium (HiMedia, Mumbai, India) in Erlenmeyer flasks for 7 days at 25 °C in the dark (static cultivation). *Aspergillus niger* (Tiegh.) strain CBS 140,837 used in the experiments was originally isolated from the mercury-contaminated soil [30]. To each of the Erlenmeyer flasks, 50 mL of Sabouraud growth medium was added. Different amounts of TiO₂ NP, 10, 20, 40, 80, 160, 320, 640 and 1280 mg were added to the growth media to obtain growth media with concentrations of 200, 400, 800, 1600, 3200, 6400, 12,800 and 25,600 mg L⁻¹ TiO₂ NP, respectively. Every concentration and a control sample with no added TiO₂ NP were tested in triplicate. Before inoculation, all of the Erlenmeyer flasks were placed in an ultrasonic bath for 15 min. Inoculation was accomplished by adding 50 μ L of *A. niger* spore suspension to the growth media. After a 7-day cultivation period, the pH of the growth media was measured, and fungal mycelia were removed, dried out at 60 °C and weighed.

For an experiment in which the rates of Aspergillus-mediated dissolution of TiO₂ NP were measured, a concentration of 400 mg L^{-1} TiO₂ NP in growth media was used. It was the highest concentration that did not reduce the dry weight of fungal mycelia significantly. Erlenmeyer flasks were filled with 50 mL of Sabouraud growth medium and 20 mg of TiO_2 NP. The flasks were then placed in an ultrasonic bath for 15 min prior to inoculation. A. niger was inoculated and grown in the same manner as in the experiment with different concentrations of TiO_2 NP. The control sample was not spiked with TiO_2 NP. The experiment was conducted in six replicates for both TiO_2 NP amended growth media and the control. After the 7-day cultivation period, the pH of the growth media was measured, and fungal mycelia were carefully removed by tweezers, dried out at 60 °C and weighed. The dry biomass was then analyzed for Ti content. Growth media were removed and centrifuged at $1000 \times g$ for one minute to separate TiO₂ NP particles and aggregates smaller than 1000 nm. After the centrifugation, 2 mL of supernatant was pipetted off and sent for the analysis of Ti content. The dissolved Ti was obtained by transferring 6 mL of supernatant that was added to ultrafiltration centrifugation units (Sartorius Vivaspin[®], Göttingen, Germany, 6 mL, 3 kDa) and the units were then centrifuged for 20 min at $3500 \times g$. Afterwards, 1.5 mL of filtrate was removed to analyze the sample for Ti content.

2.4. Determination of Ti Concentration by ICP-MS

The collected supernatants, filtrates and the dry biomass were digested in the mixture of HNO₃ and HF acids by microwave digestion at 1200 W for 30 min (Anton Paar Multiwave 3000, Anton Paar, Graz, Austria). The digested samples were analyzed for Ti content by ICP-MS (SCIEX ELAN 6000, Perkin-Elmer SCIEX, Quebec, Canada). The isotope of ⁵⁰Ti was used to collect the data and ¹⁰³Rh was used as an internal standard.

2.5. Statistical Analysis

Concentrations of Ti in dry biomass and growth media, the dry weight of mycelia and pH were compared for all treatment conditions via two-tail *t*-test at a significance level $\alpha = 0.05$. Prior to *t*-test, data were analyzed for differences in variances by *F*-test and then either *t*-test for equal or *t*-test for unequal variances was used. The statistical evaluation was completed using Analysis ToolPak add-in for Microsoft Excel (Redmond, WA, USA).

3. Results

3.1. Batch Experiment with Soil

After being treated with TiO_2 NP, two soils of different pH values, alkaline agricultural Calcic Chernozem (CH-cc) and strongly acidic forest Dystric Stagnic Cambisol (CM-st.dy) (Table S1) were studied for the distribution of mobile Ti in aggregates in different particle size ranges. A substantial difference in soil pH was selected deliberately to investigate the extent to which a pH affects the TiO_2 NP dissolution.

Distribution of colloidal and ionic forms in mobile Ti fraction was measured in aqueous suspensions of untreated (TiO₂ NP-free soil) and TiO₂ NP-treated soils (contaminated soil). These experimental results can be seen in Figure 1, which shows the concentrations of Ti in different particle size fractions and the retention of TiO₂ NP in CM-st.dy and CH-cc soils.

The concentration of dissolved or ionic Ti in untreated soils was below the 0.005 mg L⁻¹ limit of quantification (Figure 1a) and only up to 0.12 mg L⁻¹ of Ti was associated with particles in 1–100 nm size range (Figure 1b). Most of the Ti was found in colloidal fraction with the highest volume of Ti bound to particles or aggregates within a 450 to 1000 nm size range (Figure 1d,e). The fraction of particles sized 100–450 nm contained the second-largest amount of Ti (Figure 1c,e).

In the TiO₂ NP-treated soils, a concentration of an ionic Ti reached 0.010 and 0.005 mg L⁻¹ in CH-cc and CM-st.dy suspensions, respectively (Figure 1a). A large portion of Ti was bound to particles

and aggregates sized between 1 to 100 nm (Figure 1b,e,f) and to those in a 100–450 nm size range (Figure 1c,e,f). Lesser amounts of TiO_2 NP were accumulated in 450–1000 nm size fraction of soil suspension (Figure 1d,e,f). Less than 30% of added TiO_2 NP was retained in both soils (Figure 1g).



Figure 1. Concentrations (**a**–**d**) and distribution (**e**,**f**) of Ti in various particle size fractions in untreated and treated soil suspensions and the retention of Ti in contaminated soils (**g**). Lower-case letters indicate no significant differences between the bars that represents soils and their different treatments (p < 0.05).

3.2. Mutual Interactions of Fungus and TiO₂ NP

Various doses of TiO₂ NP were supplemented into the growth media to evaluate the fungal growth response to nanoparticles (Figure 2). The addition of TiO₂ NP, up to a concentration of 400 mg L⁻¹, caused no statistically significant weight changes in fungal biomass. The pH of the nanoparticle-amended growth medium was similar to that of TiO₂ NP-free medium. However, concentrations over 800 mg L⁻¹ were found to have statistically significant negative effects on the development of *A. niger* and reduced the dry weight of the mycelia. Hence, the 400 mg L⁻¹ TiO₂ NP concentration was selected for further experiment.



Figure 2. Growth of *Aspergillus niger* at different TiO₂ NP concentrations. * indicates significant differences between treatments and control (p < 0.05).

At an initial TiO₂ NP concentration of 400 mg L⁻¹, fungus *A. niger* acidified its environment (i.e. growth medium) to a value as low as 2.5. The fungus accumulated 3.6 mg g⁻¹ of Ti in the dry mycelia. The remaining Ti was mainly associated with aggregated sediments. Approximately 90% of the TiO₂ NP settled during cultivation (Figure 3), while 7.5% were bioaccumulated or incorporated into the growing biomass mechanically. Less than 2.5% remained in the form of particles and aggregates with a size up to 1000 nm and only 0.01% (0.030 mg L⁻¹) was dissolved in the growth media during cultivation.



Figure 3. Distribution of Ti in the cultivation system after incubation of *A. niger* in the presence of 400 mg L^{-1} TiO₂ NP.

4. Discussion

4.1. Batch Experiment with Soils

Our results suggest that the properties of alkaline agricultural soil and properties of an acidic forest soil affect the distribution of TiO_2 NP in soil suspension in a similar way (Figure 1f). If pH was the only factor affecting the TiO_2 NP distribution, this finding would be quite unexpected (lower TiO_2 NP retention would be expected for CH-cc compared to CM-st.dy) [47]. Since both soils differ significantly in clay and organic carbon content, we hypothesize that two concurrent factors affected the observed behaviour: (1) Humic acids stabilize nanoparticles and their aggregates by adsorbing onto their surfaces. This process is accompanied by the development of a negative surface charge which leads to the stabilization of the nanoparticles in a wide soil pH range [48–50]. (2) High content of clay and silt fraction in CH-cc provides a larger surface area on which TiO_2 NP can be easily attached to or TiO_2 NP can make aggregates with this soil fraction and, thus, remain suspended in soil solutions [51,52]. Therefore, even though the pH values differ significantly, the combined effect of the two factors mentioned above results in a similar size fractionation of Ti in both soil solutions.

Some level of homoaggregation of TiO_2 NP may have also occurred [53] and it may have resulted in a higher content of Ti detected in 1–100 nm and 100–450 nm size fractions in TiO_2 NP-treated soils (Figure 1e,f). Only a negligible amount of Ti from the TiO_2 NP was released into solution in both soil types studied. This is in line with observations where even in very acidic soils, the Ti dissolution is minimal, although it does occur naturally [3].

Compared to real soil conditions, soil batch experiments cannot simulate some factors that influence the transport of nanoparticles in soil. Under natural conditions, soils have a complex structure with micro- and macropores. Moreover, soil organisms and plants help to form additional pores in soil. These factors heavily affect the porosity and hydraulic conductivity, and, thus, the distribution of nanoparticles in the soil environment. Failing to account for these factors can lead to both underestimation and overestimation of the nanoparticle mobility in soils [21,27,54].

4.2. Fungal Accumulation of TiO₂ Nanoparticles

Microscopic filamentous fungi mechanically and chemically disintegrate soil particles and fasten the weathering process [55]. However, Ti minerals are one of the most stable solid soil phases [4]. The accumulation of Ti in fungi or plants should thus be minimal. Even with the biologically enhanced mobilization induced by acidolysis and complexolysis, only a negligible amount of the dissolved Ti is released to the soil solutions [4,5,28,56].

A. niger acidified its environment to pH 2.5. It has been reported that under such conditions, the fungus also releases large amounts of organic acids and other biomolecules that enable the chelation and complexolysis [29,31,57]. However, in our experiment, only 0.01% of the initial content of Ti was detected in growth media in a solubilized form (Figure 3). Thus, we hypothesize that *A. niger* strain's mycelia accumulated most of Ti in the form of TiO₂ NP aggregates. However, the design of the experiment has not allowed for an accurate estimation of the volume of TiO₂ NP that has been accumulated on the mycelial surface and the amount of TiO₂ NP that have been absorbed. Likewise, we do not know whether Ti present in the fungus was first dissolved and then absorbed and bound to organic molecules in fungal cells or if *A. niger* reprecipitated the dissolved portion of Ti as biogenic TiO₂ NP inside its cells. Such bioaccumulation of TiO₂ NP may affect the spatial distribution of Ti asoils, and soil fungal biomass can act as a source of Ti for other organisms. The concentration of Ti accumulated in the mycelia (3.6 g kg⁻¹ dry weight) was higher than Ti concentration in roots of barley grown in the hydroponic system (0.26 to 1.49 g kg⁻¹ dry weight) at TiO₂ NP content of 100 to 1000 mg L⁻¹ [12].

The aggregation of TiO_2 NP was also accompanied by the settling of agglomerated particles to the bottom of the Erlenmeyer flask; the aggregation was likely enhanced by secretion of extracellular polymeric substances by *A. niger*. These exudates attach to nanoparticle surfaces and promote aggregation via alteration of surface properties [58–61].

5. Conclusions

Our results indicate that *Aspergillus niger* strain and other similar soil fungi are organisms capable of redistributing Ti in soil horizons. They can bind relatively high amounts of TiO_2 NP into their mycelia. Soil fungi and other soil microorganisms able to produce organic and inorganic acids should be considered when evaluating distribution and environmental risks of Ti and other elements conventionally referred to as immobile since they contribute to the accumulation and redistribution of these elements in soils. Future research should refine the estimate of the amounts of Ti attached to mycelial surfaces and Ti accumulated in the mycelia using more precise methods. Moreover, since *A. niger* has a potential to dissolve and remineralize TiO_2 NP, laboratory experiments able to adequately capture this process would provide unique insights into the behaviour of fungus during bioaccumulation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/11/1833/s1, Table S1: Characteristics of Calcic Chernozem (CH-cc) and Dystric Stagnic Cambisol (CM-st.dy) soil samples.

Author Contributions: Conceptualization, L.N., M.Š. and M.U.; data curation, M.Š.; formal analysis, M.Š.; funding acquisition, M.U. and P.M.; investigation, M.Š.; methodology, M.Š., M.U. and P.M.; project administration, P.M.; resources, M.K., M.B. and I.H.; supervision, M.U., M.K. and P.M.; validation, M.B.; visualization, M.Š.; writing—original draft, M.Š.; writing—review and editing, L.N. and M.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences (Vedecká grantová agentúra MŠVVaŠ SR a SAV) under the contracts Nos. VEGA 1/0153/17, VEGA 1/0164/17, and VEGA 1/0146/18. And by Cultural and Educational Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic (Kultúrna a edukačná grantová agentúra MŠVVaŠ SR) under the contract KEGA 013SPU-4/2019.

Conflicts of Interest: The authors declare no conflict of interest.

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