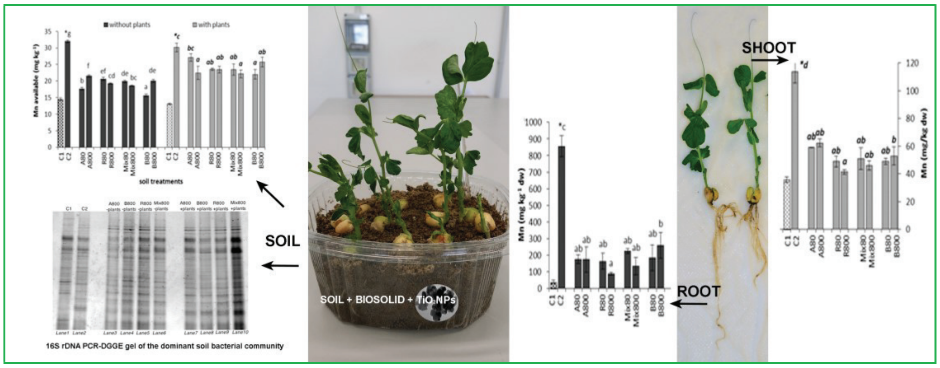
Graphical Abstract



Highlights

- TiO_2 treatments in soil induced the depletion of mineral nutrients availability
- A dose-dependent reduction of bacterial biodiversity was observed in treated soils
- A general imbalance of pea mineral nutrition was observed in TiO_2 treated soils
- No evident effect attributed to a particular crystalline phase was observed
- The Mix of anatase and rutile seems to be more deleterious in the soil-plant system

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TiO_2 nanoparticles in a biosolid-amended soil and their implication in soil nutrients, microorganisms and plant nutrition

Lorenza Bellani [a,b], Giovanna Siracusa [c], Lucia Giorgetti [b], Simona Di Gregorio [c], Monica Ruffini Castiglione [c], Carmelina Spanò [c], Simonetta Muccifora [a], Stefania Bottega [c], Roberto Pini [d], Eliana Tassi [d]*

[a] Department of Life Sciences, University of Siena, via A. Moro, 2 - 53100 Siena, Italy

[b] Institute of Agricultural Biology and Biotechnology, National Research Council (IBBA-CNR), via G. Moruzzi, 1 - 56124 Pisa, Italy

[c] Department of Biology, University of Pisa, via L. Ghini, 13 - 56126 Pisa, Italy

[d] Research Institute on Terrestrial Ecosystems (IRET-CNR), via G. Moruzzi, 1 - 56124 Pisa, Italy

* corresponding author – email address: elianalanfranca.tassi@cnr.it

Abstract

The wide use of nanoparticles (NPs), gives concern about their possible negative implications in the environment and living organisms. In particular, titanium dioxide (TiO_2) NPs are accumulated in biosolid (Bs) from wastewater treatment plants, which is used as amendment in farm soils, becoming an important way of NPs entrance in the terrestrial ecosystems. In this study, to simulate a low and cumulative load of NPs, 80 and 800 mg TiO_2 kg^{-1} of soil were spiked in the Bs prior its addition to soil. The effects of TiO_2 NPs (pure anatase and rutile or their mixture) and the bulk counterpart on the availability of mineral nutrients and bacterial communities of treated soils, together with the nutritional status of *Pisum sativum* L. plants were evaluated. Results showed the reduction, to different extents, on the availability of important soil mineral nutrients (e.g. Mn -65%, Fe -20%, P -27%, averagely), in some cases size- (e.g. P) and dose-dependent. Bacterial communities were also affected by the presence of TiO_2 particles in soil, being their biodiversity most reduced by the high TiO_2 dose. The mineral nutrition of pea plants was also altered, showing the main reduction in Mn (80% in the roots and 50% in the shoots), K, Zn, P (80, 40, 35% in roots, respectively), and an increase in N, with possible consequences on the quality of the crop. The present study gives new integrated data on the effects of TiO_2 NPs in the soil-plant system, on the soil health and the nutritional quality of crops, rising with new implications for future policies and human health.

Keywords: titanium dioxide nanoparticles; anatase and rutile; bacterial communities; mineral nutrients; *Pisum sativum*; terrestrial environment

1 Introduction

Engineered nanoparticles (ENPs) find numerous growing applications to a broad range of industrial sectors such as cosmetics, coatings and paints, plastics, foods and beverages, pharmaceutics, textiles, environment, electronics, transports, etc. (Keller et al., 2013; Piccinno et al., 2012; Roco, 2011). However, the ambiguous behavior of ENPs, i.e., benefits vs. negative implications for the society, environment and economy systems (Tolaymat et al. 2017), gives concern about their increasing release and effects on the environment.

A survey from companies producing or using ENPs revealed TiO_2 as the most produced ENP with up to 50,000 t/year of worldwide production (Kunhikrishnan et al., 2015). Modeling studies providing predictions for the TiO_2 nanoparticles (NPs) production, application and release indicated them among the most used NPs and the ones with far higher concentrations in all environmental systems (Hendren et al., 2011; Sun et al., 2014). Significant flows of TiO_2 NPs from wastewater treatment plants (WWTP) were predicted to be discharged to sewers, accumulated in biosolid (Bs) and wastewater effluents, which will end up in farm soils (8900 t/year), landfills (7600 t/year) or in the water systems (3600 t/year) (Garner et al., 2017; Nowack et al., 2016; Song et al., 2017). Soil ecosystems (natural, farm, urban, industrial and landfill soils) are considered important sinks for the ENPs (Judy and Bertsch, 2014; Keller et al., 2013), in particular, farm soils, where the application of Bs and sewage sludge as fertilizers becomes a significant route of entry in the terrestrial environment (Jesmer et al., 2017; Judy et al., 2015; Ma et al., 2014). Moreover, the uptake of NPs by edible plants could represent a way of transfer into the food chain (Pošćić et al., 2016; Servin et al., 2013; Tan et al., 2018).

The production of wastewater sludge and Bs in EU was estimated at 10 million tons (dry solids) in 2008, 44% of which recycled to land, and, by 2020, it is expected to reach a total production of 13 million tons (EC Overview Report, 2008). The use of Bs in agricultural soils is currently identified as one of the best environmental management practice, due to the supply of organic matter and nutrients to the soil-plant system. The EU Directive 86/278/EEC seeks to boost its use in agriculture and its quality control to prevent harmful effects to humans and environment. Accordingly, Bs and sewage sludge designated to farm soils are subject to concentration limits for toxic metals, organic molecules and pathogens, however, no restrictions are defined for the presence of ENPs, although increasing concern regarding their accumulation and ecotoxicity in long-term land application have been found (Chen et al., 2017; Choi et al., 2017; Dulger et al., 2016; Eduok et al., 2017; Yang et al., 2014).

The effects of ENPs on plants will depend on a series of factors (NPs type, plant species, soil properties, etc.). In particular, the soil organic matter could influence the mobility and the bioavailability of ENPs by changing their original properties such as aggregation state, surface charge affinity, Van der Waals force and zeta potential (Pošćić et al., 2016; Tan et al., 2018; Tassi et al., 2012). Although several studies investigated the accumulation and effects of TiO_2 NPs on different plant species (Deng et al., 2017; Larue et al., 2016; Ruffini Castiglione et al., 2014; Ruffini Castiglione et al., 2016), the ones conducted in real farm soils (Pošćić et al., 2016; Tassi et al., 2016) are yet not conclusive. Moreover, the evaluation of NPs' impact on soil microbial communities (Chen et al., 2017; Judy et al., 2015; Simonin et al., 2015) or on mineral nutrient availability (Dimpka et al., 2015; Pošćić et al., 2016) are still scarce and limited. Furthermore, TiO_2 crystalline phases, anatase and rutile, were found to distinctly affect physiological, biochemical and genotoxic plant parameters (Tan et al., 2018). Anatase in plants was reported to be more toxic than rutile: Silva et al. (2016) revealed higher anatase toxicity on wheat seeds germination and increased membrane permeability than the mixture of anatase and rutile; Giorgetti et al. (2019) showed that anatase on its own or mixed with rutile induced higher oxidative stress and ultrastructural damages in roots of pea plants than rutile; Cai et al. (2017) showed a preferential translocation of anatase from the roots to the upper part of rice plants when a mixture of anatase and rutile was present. However, a preferential uptake of rutile in cucumber plants exposed to a mixture of anatase and rutile was demonstrated by Servin et al. (2012). Therefore, more information about the toxicity, in soil-plant system, of the different crystalline phases and their entry into food crops are important and necessary, in particular, for NPs coming from a Bs amended farm soil.

In this study, it was hypothesized that anatase and rutile crystalline forms, as well as larger TiO_2 particles (here named as bulk) may have different and specific influence on the soil-plant system in terms of mineral nutrients availability and soil microbial community, as well as on the nutritional status of plants. To assess these hypotheses, this work evaluated the effects of TiO_2 as anatase and rutile NPs (separately and mixed together) and as bulk particle on the availability of mineral nutrients in a biosolid-amended farm soil, on the disturbance of associated soil bacterial community and on the mineral nutrition of a crop plant *Pisum sativum* L., simulating a low and a cumulative TiO_2 load through Bs application in the agro-ecosystem.

2 Material and Methods

2.1 Characterization of *pristine nanoparticles*

Commercial powder of TiO_2 NPs was bought from US Research Nanomaterials Inc. (Houston, USA) as anatase or rutile crystal phases (both with a nominal size of 30 nm) and non-coated bulk TiO_2 particles from Sigma-Aldrich (Saint Louis, USA, size > 100 nm), all having at least 99.8% of purity (producers' information). Morphology and size of TiO_2 were characterized by Transmission Electron Microscope (TEM, FEI Technai), placing a drop of 80 mg L^{-1} suspension on grids covered by formvar, allowed to settle and observed at 100 kv. Purity was determined assessing the recovery of Ti on the TiO_2 particles analysed by Inductively Coupled Plasma Optical Emission Spectrometry

(ICP-OES, Varian Liberty Axial) after a two-step digestion method in an open-block digester as described in Fang et al. (2009) and Giorgetti et al. (2019).

2.2 Soil, biosolid amendment and growth matrix preparation

The agricultural soil used as control (C1) was obtained from the Agri-Environmental Research Center 'Enrico Avanzi' (CiRAA - University of Pisa, Italy). This soil was characterized by sandy texture (sand 93.3%, clay 2.1%), organic matter (OM) of 1.1% and pH of 7.7. Biosolid was obtained in the cake form at a municipal WWTP near Pisa (Italy), it consisted of 18% solid residue (105°C) with 57.3% of OM and complied the Italian law for the disposal in farm soils (Legislative Decree n°99/92). Soil analysis were performed using the standard methods (SSSA, 1996): sand, silt and clay contents were determined by pipette method, cation exchange capacity (CEC) by barium chloride method; electrical conductivity (EC) and pH by the appropriate electrodes in a soil/water ratio of 1:2.5. Elementary C and N were analyzed by dry combustion using, respectively, the Multiphase Carbon Determinator (LECO RC-412) and the Nitrogen/Protein Analyzer (LECO FP-528); OM was calculated from the content of organic C. The total content of mineral nutrients (P, K, Ca, Mg, Fe, Mn, Cu, Zn) was determined after a single-step digestion method (H_2O_2/HNO_3 ratio 1:2.5, v/v, EPA method 3051-A, 1995) in a microwave oven (FKV ETHOS 900). Instead, for the total content of Ti, a two-step digestion method in an open-block-digester was used. Elements were analyzed by ICP-OES.

For the preparation of growth matrixes, nano anatase, nano rutile or bulk TiO_2 particles were suspended in ultrapure milli-Q water (18 Ω cm^{-1}, Merck Millipore) by sonication (Sonifier 250, Branson) for 30 min in continuous mode and an output power of 80W. For the Mix, pristine anatase and rutile TiO_2 NPs were suspended together in a 1:1 ratio, in order to observe the eventual effect of the simultaneous presence of both crystal phases with no influence of their concentration.

Suspended TiO_2 particles and plain milliQ water were then vigorously mixed with the Bs cake during 24h for the spiked and the non-spiked Bs preparation, respectively. The obtained Bs-slurries were exposed for 30d to ambient environmental conditions to allow the possible transformations or aging of pristine TiO_2 particles in the Bs, thus supposing that particles stored or otherwise transformed could behave differently than pristine particles. Bs spiked with TiO_2 and that non-spiked were then thoroughly mixed with the control soil (C1) soil in a Bs:C1 ratio of 3:100, on dry-weight (dw) basis, and left to open-air to permit equilibration and further reactions with soil components.

2.3 Set up of pot trials

Two different concentrations of TiO_2 particles were used in the experiment, so that the nominal concentrations of nano or bulk TiO_2 particles spiked in soil were calculated to be 80 and 800 mg of TiO_2 kg^{-1} of soil. These concentrations corresponded to low and cumulative load of TiO_2 particles in the farm soil receiving the Bs amendment (Sun et al., 2014). Ten different treatments were designed and named as follows:

C1 = control soil;

C2 = amended soil control;

A80 and A800 = amended soil spiked with Anatase NPs;

R80 and R800 = amended soil spiked with Rutile NPs;

Mix80 and Mix800 = amended soil spiked with a mixture of both pristine NPs, in a ratio 1:1 Anatase:Rutile;

B80 and B800 = amended soil spiked with Bulk TiO_2 particles.

Five pots per treatment were filled with the growth matrices, each with 500 g (dw). Four pots per treatment were sowed, each with 10 previously hydrated seeds of *Pisum sativum* L. and one pot per treatment remained seed free. All pots were disposed randomly at controlled conditions of light (16/8 h day/night photoperiod), temperature (22/18 °C day/night) and relative humidity (65-70%), maintained by watering with tap water during the growth.

2.4 Plant and soil analysis

After 28 days, plants were harvested, roots and shoots separated and carefully washed. Soil particles possibly adhered to the roots were further eliminated by sonication in deionized water using a pulse mode and an output power of 15W for about 5 min. The plant growth was estimated by measuring the length of both roots and shoots and their biomasses after oven-drying at 40°C until constant weight. Pigments (total chlorophyll and carotenoids) were extracted from fresh leaves in 80% acetone and their amount determined according to Hassanzadeh et al. (2009) and Lichtenthaler (1987), respectively. Dried roots and shoots were separately grounded to fine powder, acid digested and analyzed for the content of mineral nutrients. Digestion methods in plants were the same used for the total elements in soils (EPA method 3051-A, 1995). The bioavailable fraction of mineral nutrients in soils (P, K, Ca, Mg, Fe, Mn, Cu, Zn) was determined by single step soil extraction from the pots with and without plants after their harvesting. Specific extracting agents were used: ammonium acetate (1M NH_4OAc at neutral pH) for the available K, Ca and Mg; sodium bicarbonate (0.5M $NaHCO_3$ at pH 8.5) for the available P; and a DTPA solution (0.01M at pH 7.3) for the available Fe, Mn, Cu and Zn. Moreover, micronutrients (Fe, Mn, Cu, Zn) and Ti were also extracted from soils with a diluted calcium chloride solution (0.01M $CaCl_2$). The DTPA extraction enables the assessment of metals and nutrients potentially available to plants (Bretzel et al., 2018), while the extraction in 0.01M $CaCl_2$, enables to determine the elements immediately available to plants, simulating soil porewater (Houba et al., 2000). Elements in the different soil extracts and in the digested plants were analyzed using ICP-OES, except for P that was determined by azomethine colorimetric method (SSSA, 1996).

2.5 Soil bacterial community analysis by molecular techniques

After plants harvesting and careful roots removal, soil samples from rhizosphere and from un-vegetated pots were collected and preserved at -20 °C until analysis. Standard procedures were used for the nucleic acid manipulation and the analysis by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Soil DNA was extracted by using the Ultraclean™ Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). DNA was manipulated using enzymes purchased from Sigma-Aldrich (Milan, Italy). The V3 region (position 341–534, *Escherichia coli* numbering) of gene encoding the bacterial 16S rRNA was amplified by PCR using the primers p3/p2 (Muyzer et al., 1993). The PCR products were separated on polyacrylamide gels [8 % (w/v), 37.5:1 acrylamide–bisacrylamide] with a 30–60% linear gradient of urea. Denaturing gels were run using the DCode™ Universal Mutation Detection System (Bio-Rad, USA). The gel images were acquired using the ChemiDoc Gel Documentation System (Bio-Rad, USA). DGGE profiles, concerning the presence and intensity of the bands, were analyzed using Quantity One (Bio-Rad, USA) to calculate the Shannon's diversity (H) and the Evenness indexes (E) (Pielou, 1975). A pairwise distance matrix was calculated and analyzed with weighted pair group main average (WPGMA) cluster analysis and presented as dendrograms.

2.6 Quality control and statistical analysis

Quality assurance and quality control for the analysis of elements on ICP-OES were performed by testing two standard solutions every 5 samples. Certified reference material (SQC-001, Metals in soil) was used to control the quality of the analytical system. Statistical analysis was performed using the Statistica package (StatSoft) version 6.0. Two-way ANOVA and a Tukey *post-hoc* analysis were performed to evaluate the significant differences for the treatment effects (A, R, Mix, B), for the two doses (80 and 800 mg kg^{-1}) and for their interaction. At least three replicates from three independent experiments were compared at $p < 0.05$. T student test was used to evaluate the significant differences among the control (C1) and amended control (C2) samples. The significance of 16S rDNA PCR-DGGE results was tested using the Bonferroni correction.

3 Results

3.1 Nanoparticles characterization

Under TEM both TiO_2 NPs (Fig. SI1 a-b) appeared highly aggregated with prismatic shape: the anatase NPs showed sizes varying from 20 to 80 nm (Fig. SI1a) and the rutile NPs had road-like shape with cusps and sizes varying from 20 to 25 nm in the minor axis and from 30 to 100 nm in the major one (Fig. SI1b). The bulk material appeared as larger aggregates of near spherical particles with sizes varying from 100 nm to 300 nm (Fig. SI1c). The purity of the material was assessed by analysing Ti in TiO_2 particles and the mean recovery as TiO_2 was 99.83 ± 2.16 (n=5 ± sd).

3.2 Composition of control, amended control and TiO_2 spiked soils

In Table 1 the chemical-physical properties of the control soil (C1), the Bs and the amended soil control (C2) were reported together with the total content of mineral nutrients (P, K, Ca, Mg, Fe, Mn, Cu, Zn) in each matrix. When compared to the C1 soil, a significant increase of important soil quality parameters such as OM (+209%), EC (+162%) and CEC (+51%), were observed in the C2 soil, as well as a huge increase of mineral nutrients, in particular Cu (+627%), N_{tot} (+470%), Zn (+315%) and P (+238%). The C:N ratio changed from 15.1 (C1) to 8.7 (C2).

Total Ti content in the soils was shown in Table 2. A high total Ti concentration of 1529 ± 152 mg kg^{-1} was observed in the C1 soil and of 699 ± 105 mg kg^{-1} in the Bs. Therefore, the addition of Bs to the C1 soil end up with the Ti value of 1667 ± 127 mg kg^{1} in the C2 soil, which is however not significantly different from C1. The spiking with low dose of TiO_2 particles determined no significant differences in the total Ti content, while the spiking with high dose showed a significant increase of the total Ti content in respect to the control (C1) and the amended soil control (C2), reaching Ti values higher than 2000 mg kg^{1}. Notwithstanding this, Ti concentration in the soil porewater, i.e. the fraction immediately available for plants and obtained with diluted $CaCl_2$ soil extraction, was below the detection limit of the ICP-OES.

3.3 Influence of TiO_2 particles in the availability of soil nutrients

The analysis of mineral nutrients in the soil porewater revealed detectable concentrations only for Mn (Fig. 1a), whereas in the fraction potentially available for plants all the nutrients analyzed were measurable (Fig. 1b-d and Fig. SI2). A huge increase of the available mineral nutrients in the C2 soil (without and with plants) in respect to C1, due to the supply of nutrients by the Bs amendment, was observed. However, an exception was detected for Ca (Fig. SI2b), which was significantly reduced in the C2 soil (about 16%) respect to C1.

The amendment of soils with Bs spiked with TiO_2 particles changed significantly the availability of Mn, Fe and P (Fig. 1), the former showing a mean reduction of about 65% in the soil porewater (Fig. 1a) and of about 40% in the potentially available fraction (Fig. 1b) in the treated soils without plants, in respect to C2. In both fractions, differences were significant in function of Ti doses (Table SI1a), particularly evident for A80 and B80, Mn being reduced in respect to the corresponding high doses by 21 and 42% in the soil porewater (Fig. 1a) and by 18% and 22% in the potentially available fraction (Fig. 1b), respectively. Significant differences were also observed for both Mn fractions in function of treatments (Table SI1a), R800 and Mix800 being reduced in respect to C2, by about 74% and 33-37%, respectively for soil porewater and potentially available fractions (Fig. 1a,b). In the soils with plants, Mn was averagely reduced to a lesser extent than the soils without plants, about 30% in the soil porewater (Fig. 1a) and about 18% in the potentially available fraction (Fig. 1b), in respect to C2, with significant differences in function of doses (Table SI1a). Manganese in A800 was reduced by about 25 and 17%, respectively in soil porewater and potentially available fractions, while in B800 an increase of about 39% in porewater, respect to the corresponding low doses (Fig. 1a,b) was found. On the contrary, non-significant differences in function of treatments (Table SI1a) were found in both fractions of soil with plants.

The reduction of Fe availability (Fig. 1c) was significant in function of dose without and with plants (Table SI1a), particularly evident in R800 and Mix800 without plants, about 20% respect to C2 and 8-9% respect to the corresponding low Ti dose treatments. Significant reduction in function of treatments was also found in soil with plants (Table SI1a).

Phosphorus availability (Fig. 1d) showed significant reductions in function of doses and treatments (Table SI1a). In the soil without plants, P in R800 and A800 was reduced by about 28 and 24%, respectively, in comparison to the low dose, and in R800, A800 and Mix800 by about 32, 25 and 12%, respectively, in comparison to C2. Instead, the treatment with bulk TiO_2 induced no significant variation on the availability of P in respect to C2 and between the doses. However, in soils with plants, only A80 treatment induced a significant reduction on P availability (about 12%, respect to C2).

A general low variation of K, Ca, Mg, Cu and Zn content in presence of TiO_2 spiked was observed (Fig. SI2), with some significant differences particularly evident for the soils without plants: K availability (Fig. SI2a) significantly increased in function of dose, treatment and interaction (Table SI1a), as in Mix80 (+23% in respect to Mix800, +30% in respect to R80 and

+41% in respect to R800); Ca availability (Fig. SI2b) showed a significant difference in function of dose and interaction (Table SI1a), as in Mix800 with a decrease of -17% in respect to Mix80; Mg availability (Fig. SI2c) significantly increased in function of treatment (Table SI1a), Mix80 being +27% in respect to A80; Cu availability (Fig. SI2d) significantly increased in function of dose, treatment and interaction (Table SI1a), as in R80 and Mix80 (11-13% in respect to C2, B80 and A80); finally Zn availability (Fig. SI2e) showed a significant increase in function of dose and interaction (Table SI1a), as in R80 (+7% in respect to C2 and Mix800). For soils with plants, only K availability was significantly reduced in function of dose in A800 (-17% respect to C2).

3.4 Influence of TiO_2 particles in soil microorganisms

Molecular profiles of bacterial community in the treated soils were reported in Table 3, as Shannon Weaver (H) and Evenness (E) indexes, obtained in soils without and with plants, for both conditions of low (Table 3a) and high (Table 3b) TiO_2 doses. An increase in bacterial biodiversity in the amended control soil (C2) was evidenced by H index higher than in the control soil (C1). In parallel, it was observed the absence of dominant populations in the microbial communities of both soils, indicated by the same values of E index. Moreover, the dendrogram (Fig. SI3) evidenced that the microbial communities of both, control and amended control soils, had a similar taxonomic nature of microbial community but differently grouped from those of TiO_2 treated soils, in which a selection of specific group of bacteria in response to the presence of TiO_2 in soil was observed. In fact, generally low doses of TiO_2 (Table 3a) induced a biostimulation of the bacterial community, as evidenced by an increase of both H and E indexes, respect to the C2 soil. The highest H index was observed for the treatment R80 (without plants), but it was reduced in the presence of plants. In the presence of Mix80, a very low H index was observed in the soil without plants and, on the other side, a very high H and E indexes in the soil with plants (Table 3a).

Concerning the high dose of TiO_2 particles, in A800 and B800 (without plants) a uniform biostimulation effect on the bacterial population in respect to C2 was observed, with an increase of H and no variation of E. Instead, the treatments R800 and Mix800 (without plants) induced a negative effect equally distributed among all the bacterial population, evidenced by the decrease of H and the increase of E, respect to C2. A general more deleterious effect for the soil bacterial biodiversity was observed in the presence of plants, since all treatments had the H index reduced (Table 3b), particularly evident in Mix800 with plants, which showed the lowest H and E. Similarly, the presence of plants in A800, R800 and B800 induced a decrease of the bacterial biodiversity respect to the same treatment without them, as confirmed by the tendency of segregation in distant groups in the dendrogram at the high dose treatments (Fig. SI3).

3.5 Influence of TiO_2 particles in plant growth, biomass, mineral nutrition and pigments

The length and the biomass of roots and shoots of pea plants from the different treatments were significantly reduced in the plants grown in the amended soil control soil (C2) in respect to those grown in the control soil (C1) (Fig. 2). A significant root length reduction was also found for TiO_2 treatments and doses when compared to C2 (Fig. 2a), particularly evident for A80 with about 35% decrease in respect to C2 and about 23% in respect to A800. Shoot length was influenced by treatments and interaction only for high dose, particularly for A800 and Mix800 in respect to C2 (Fig. 2a). Dry biomass, of either roots or shoots, showed the same trend as reported for the length, although differences were not significant due to the high standard deviation among the replicates (Fig. 2b).

Mineral nutrients detected in roots and shoots of pea plants were summarized in Fig. 3 and Fig. SI4. The concentration of Mn, Cu, Fe, Mg, Ca, and Zn significantly increased in the roots of plants grown on the C2 soil, in respect to those from the C1 soil. This increase was particularly evident for

Mn (Fig. 3a), while K and P content did not change (Fig. 3b,d) and N was reduced by about 10% (Fig. SI4b). Moreover, in the shoots of plants grown on the C2 soil, the concentrations of Mn, K and Zn (Fig. 3a-c), Mg and Ca (Fig. SI4a,d) increased significantly, while P (Fig. 3d), N and Cu (Fig. SI3b,c) content did not change and Fe (Fig. SI4e) was reduced by 19% when compared to the C1 soil.

However, TiO_2 treatments induced a general imbalance on the mineral nutrition of plants in respect to the amended soil control (C2), Mn, K, Zn and P being the elements mostly reduced, particularly in the root compartment (Fig. 3). Among these nutrients, Mn was averagely reduced by 80% in the roots, significant in function of dose and in particular for R800 and Mix800 (about 40% less than the respective low dose). Manganese in the shoots (Fig. 3a) showed an average reduction of 55%in respect to C2, significant in function of dose and treatment (Table SI1b), and particularly evident for R800 (-16% in respect to R80 and -34% in respect to A800).

Significant decrease of K was observed in the pea roots (about 80%, Fig. 3b), significant in function of dose, treatment and interaction (Table SI1b), with the exception of bulk treatment. No significant differences were observed for K in the shoots of all treatments (Fig. 3b). Moreover, a significant reduction, in function of Ti dose (Table SI1b), was observed in both roots and shoots tissues for Zn (-40 and -14%, respectively, Fig. 3c) and P (-35% in both tissues, Fig. 3d), in respect to C2. In addition, Mg in the shoots from all treatments was reduced by about 18% in respect to C2(Fig. SI4a), which resulted significant for dose, treatment and interaction (Table SI1b). Nitrogen (Fig. SI4b) increasedin both, roots (averagely of 40% in A800, B80, B800) and shoots (about 8% in all the treatments with the exception of B800), which resulted significant for dose, treatment and interaction (Table SI1b).

Regarding the pigments, no significant differences on both total chlorophyll and carotenoids content was observed in the shoots of pea plants (Fig. 4) grown on the C1 and C2 soils. Moreover, the treatments with TiO_2 induced a general non-significant alteration of total chlorophyll content, in function of dose and treatment (Fig. 4). For carotenoids a significant effect was observed for dose and interaction, in particular for R800 and B800 in respect to the corresponding low dose treatments (Fig. 4).

Discussion

The amendment of control soil (C1) with biosolid (Bs) resulted in a non-substantial imbalance between mineralization and humification processes of soil OM. In fact, the ratio C:N observed for the control soil (C1) and the amended soil control (C2) falls within the normal ranges for agricultural soils (Costantini and Lorenzetti, 2013). Moreover, the high CEC value indicated an increased amount of negative charges in soil (due to the increase of OM) and a greater capacity to hold cations (or mineral nutrients) of the C2 soil in respect to C1. The amendment of soil with Bs increased the microbial biodiversity, but did not affect the relative abundances of all bacterial specimen, as observed in resilient soils (Bevivino et al., 2014; Griffiths and Philippot, 2013; Siracusa, 2018). However, the observed reduced length and biomass of pea plants grown on the C2 soil, in respect to C1, can be justified by the presence of non-humified compounds and the significant increase of total and available Cu and Zn; which induced phytotoxic effects as reported in several studies (Britto and Kronzucker, 2002; Giorgetti et al., 2019; Wen et al., 2002; Zubillaga and Lavado, 2006). Nevertheless, the general increase of nutrients observed in the roots and the shoots of pea plants was in accordance with the expected effect of Bs amendment in soil, which brought an increase of available nutrients in soil for the plants uptake (Tontti et al., 2016). However, an opposite effect was observed for Ca that was reduced by the amendment, in respect to the C1 soil. This could be due to the involvement of part of Ca^{2+} ions on the stabilization of soil OM and the formation of soil micro-aggregates, in which the Ca^{2+} ions acted as inorganic binding agents through the establishment of complexes of clay-polyvalent cation-organic matter (Rowley et al., 2018; Six et al., 2004).

Ti is an intrinsic component of soil minerals, being reported as the ninth most abundant element in the earth' crust (Buettner and Valentine, 2012). The high total concentration of Ti observed in the control soil (C1) was in line with the Ti background values reported for sandy soils (Pais and Benton Jones, 2000), while the amount of Ti in the Bs was consistent with the values from different WWTPs, of 229-914 mg kg^{-1} (Josko and Oleszczuk, 2013) and 69-4510 mg kg^{-1} (Kim at al., 2012). Model predictions also reported values of Ti in biosolid (from TiO_2 NPs) ranging from 150 to 564 mg kg^{-1} (Sun et al., 2014). In our study, the addition of Bs and the TiO_2 spiked at low dose resulted no significant different in the total Ti content. Instead, TiO_2 particles spiked at high dose showed a significant increase of total Ti content, independently of the crystal phase and size. Although the high amount of total Ti in the soils, the Ti fraction immediately available for plants (Ti in soil porewater) was below the detection limit of the ICP-OES. Moreover, as reported in Giorgetti et al. (2019), the Ti fraction potentially available for plants (Ti extracted with DTPA) was very low, representing a maximum of 0.13 and 1.3% of the low and high concentrations of the Ti spiked. These results suggested that most of the spiked TiO_2 particles (in the form of nano or bulk) were entrapped (adsorbed and/or precipitated) in the soil solid phase. This high retention in our treated soils could be explained by a physical straining process (Conway and Keller, 2016), or by the formation of hetero/homo aggregates of TiO_2 NPs with reduced mobility in soils in function of the matrix composition (clay, OM, free ions content, pH) (Fang et al., 2009, Tan et al., 2018, Tassi et al., 2012). Indeed, the properties of pristine particles (surface area, zeta potential, surface affinity) could be highly subjected to physical transformations in the WWTPs (Wu et al., 2018), which could affect, to different extents, the availability of TiO_2 in soil and to plants. This phenomenon was attributed, by several authors, to the predominant functional groups of the organic molecules from humic and non-humic fractions coating the TiO_2 NPs (Fisher-Power and Cheng, 2018; Jayalath et al., 2018; Tan et al., 2018; Yang et al., 2009; Yang et al., 2014; Wu et al., 2018). Generally, the functional groups such as alcohols or phenols, carboxylic acids and amines will result in a hydrophilic coating on the pristine TiO_2 NPs and attraction with the soil particles (Tan et al., 2018). Likewise, in our system, the organic molecules from Bs should interact with the pristine NPs, coating them. Moreover, the non-complete maturity of the Bs and the possible presence of non-humified compounds such as volatile organic acids, phenols and ammonia (Giorgetti et al., 2019, Zubillaga and Lavado, 2006) could easily cover the spiked TiO_2 particles and give them a sort of hydrophilic character, favoring the adsorptive behavior in the treated soils.

The effect of spiking TiO_2 particles in the amended control soil modified to different extent the availability of mineral nutrients, particularly evident for Mn. Several studies indicated that Mn oxy/hydroxides in soils can exhibit large surface areas and be highly chemically active (Gasparatos, 2013; Post, 1999), participating to cation-exchange or oxidation-reduction reactions with other active molecules such as the TiO_2 NPs. Thus, the reduction of Mn availability in the treated soils from the present study can result from the oxidation of Mn^{2+} (form available to plants) to Mn^{4+} (form less mobile in soils and non-available to plants) by the interaction with TiO_2 particles. However, pH reduction and exudates excretion due to the presence of roots (Dotaniya and Meena, 2015) could compete with the TiO_2 particles for the Mn ions, thus representing another task on the mechanism governing the effect of TiO_2 NPs in the soil-plant system. Phosphorous reduction is supported by the well-known ability of TiO_2 NPs to adsorb phosphate ions, recently reported also in Chen et al. (2015), who showed a stronger adsorption capacity of anatase than rutile in aqueous phosphate solution. Differently, in our soil system, no clear differences between A800 and R800 in soils without plants was observed, although a higher P availability was evident for Mix 800. The observed differences in respect to the aqueous system reported by Chen et al. (2015), could be justified by the presence of organic molecules (humic and non-humic molecules and root exudates) or inorganic ions (Mn, Fe) responsible for hydrophilic and hydrophobic coatings on the TiO_2 particles, which may induce different strength of TiO_2 interaction with P (Han et al., 2014; Yang et al., 2009) and also due to eventual phosphates precipitation.

Therefore, in the system studied, the nutrients availability changed to different extents in the presence of TiO_2, with a preferential interaction, in a decreasing order, for: Mn > Fe > P and being particularly evident the interaction of P with the TiO_2 NPs respect to the bulk particles. Moreover, low and high doses of TiO_2 in soils, often produced a non-uniform response, even though the 'dose-dependence' was more frequent than the 'treatment-dependence'. This was particularly true for the soils without plants, signaling that the roots activity (and associated microorganisms) could only partially mitigate the impact of TiO_2 on the nutrients availability.

Regarding the bacterial community, low doses TiO_2 treatments provoked an evident biostimulation effect, except in the presence of mixed crystalline phases (Mix80), which induced a specific stress response of a restricted bacterial specimen. This negative effect was mitigated by the presence of plants, which induced an increase of the microbial diversity (H index increased) and contributed to an equal distribution of the different bacterial specimen characterizing the soil (E index increased) in respect to the same treatment without plants and to the amended soil control. Concerning the high dose of TiO_2 particles, a general reduced microbial biodiversity with no effect on a specific bacterial population, was evident. This effect may indicates a dose-dependent toxicity, in line with the reduction of nutrients availability. In fact, plants compete with the bacterial community for the use of the bioavailable nutrients, thus amplifying the deleterious effect of high doses particles. A not strict dose-dependent effect of NPs was reported by Sun et al., 2014. These authors evidenced that NPs aggregation can vary in function of concentration, resulting in variable NP bioavailability and even toxicity to soil microorganisms. Our results are in accordance with the study of Ge et al. (2013) indicating that TiO_2 NPs interfere with soil microbial community richness and reduced diversity. The impact of TiO_2 NPs on bacteria was attributed to the adsorption to cell membrane, causing oxidative stress associated to reactive oxygen species (ROS) production and osmotic stress (Sohm et al., 2015).

Likewise, the pea plants under the influence of TiO_2 treatments, showed a general deleterious effect. The reduction in root length was observed, while an increase in shoot length was detected for the high doses of NPs. This apparently controversial impact of TiO_2 NPs in plant growth was already reported by several authors, showing induction or inhibition in function of the concentration of TiO_2 in the media (Lyu et al., 2017 and references therein). Clément et al. (2013), who indicated a tendency of bigger homo/heteroaggregates formation upon the TiO_2 NPs concentration and reduction of their penetration through the root cell membrane, also signaled increased toxicity of TiO_2 at lower concentrations in respect to higher ones. Similar to the reduction of nutrients availability in soils, a general imbalance to plant nutrition was observed, particularly evident for Mn, as a reflection of its reduced availability in the treated soils. It is worth nothing that Mn in plants plays key roles in several physiological processes; in particular, it acts as catalyst in the oxygen-evolving complex of photosystem II. Manganese deficiency frequently occurs without visual leaf symptoms, not permitting a correct evaluation of the problem in field crops, but resulting in restrictions of crop productivity and quality (Schmidt at al., 2016). In fact, in our study no reduction on the biomass of pea plants was observed, nor in the plant pigments, indicating that longer growth experiments would be important to evaluate the impact on the productivity. The observed increase of N in plants grown in treated soils may be of particular importance in respect to the plant performance and to the growth potential, indicating a better assimilation at the root level or evidencing a poor ability of translocation to the aerial part (Masclaux-Daubresse et al., 2010). On the other hand, the study of Lyu et al. (2017) consider that bulk TiO_2 could act as helpful element for crops in a context where there is a balance in availability, uptake and distribution of nutrients in the plant body. Further studies are needed using the Next Generation Sequencing Analysis, in order to clarify these aspects, especially concerning N in soil and the role of N-fixing symbiotic bacteria in presence of NPs and bulk TiO_2. Thus, the general disorder found in the mineral nutrition of pea plants can further sustain the recorded differences in root and shoot length, observed in plants from TiO_2 treatments, roots being more sensible to TiO_2 treatments, as evidenced by the major depletion of mineral nutrients (such as Mn, Zn, K and P) in respect to shoots. This is not surprising given that roots interact directly with the particles in soil and are subjected to the variation of soil nutrients availability, particularly evident for Mn. Moreover, K and P content were mainly reduced in the roots of plants growing under the NPs treatments (independently of the crystalline phase), where a less significant effect was found for the bulk treatments. This could indicate a particular damage by the NPs at the level of the absorption organ, as a consequence of a possible membrane damage induced by the uptake of the NPs (Giorgetti et al., 2019). Wang et al. (2012) showed that CuO NPs increased particularly the leakage of K in the roots of maize, suggesting major problems coming from the root membrane damages respect to the shoot. However, the processes underlying these changes need further studies to better understand the interaction mechanism between NPs and nutrients uptake. Literature data on pigment content are quite controversial, as a dose-dependent effect was often reported with an increase (Hajra and Mondal, 2017) or a decrease (Shafea et al., 2017) upon the NPs concentration, showing that other concomitant factors other than the dose or the treatments may influence their content in plants.

Conclusions

Biosolid amendment in soil produced a significant increase of important soil quality parameters, as well as the increase of mineral nutrients and their availability to plants. Moreover, Bs amendment indicates a general trophic effect on the microbial ecology with an increase in the bacterial biodiversity not accompanied by the establishment of dominant populations. It can be associated to an improvement of the resilience of the soil due to the establishment of a highly diversified microbial ecology. However, TiO_2 nano/bulk particles was shown to accumulate at high amount in the farm soil through the Bs utilized as amendment. Their adsoption/precipitation in the soil solid phase induce negative threats to the quality of both soil and crop plants. TiO_2 spiked in the Bs reduced the availability to plants of some soil mineral nutrients, in particular Mn (-65%), Fe (-20%) and P (-25%) and caused a consequent imbalance in the mineral nutrition of pea plants (e.g. reduction of Mn -80%, Zn -40%, K -80% in roots). Moreover, a dose-dependent effect of TiO_2 treatments on the microbial ecology was observed, where the low doses produced a sort of bio-stimulating effect and the high doses were more deleterious for the microbial biodiversity, as well as that observed on the bioavailability of nutrients in soil. Actually, also a treatment-dependent effect was observed and determined a deterioration of the microbial richness and diversification, particularly evident in presence of the Mix of NPs. Our results suggested that TiO_2 may indirectly affect the mineral nutrition of plants and the soil bacteria community and the availability of nutrients in the soil, sometimes in a dose dependent manner and sometimes in a size dependent manner, with some evidences for the specific effect due to the co-presence of both crystalline phase of NPs. On the other hand, the presence of plants mitigated the effects exerted by NPs on the availability of mineral nutrients; however, they can further determine a harnessing of the stress on the microbial population at high dose treatments. Consequently, the complex system Bs-soil-plant still need further investigations to determine the possible mechanism of uptake and the impact of long-term load of TiO_2 NPs in the soil microbial ecology and the in agricultural crop plants, to permit a better foresee the behavior and the fate of TiO_2 NPs in the terrestrial ecosystem. In particular, this study pose a reflection on the use of biosolid from WWTP, in view of the emergent contaminants, as are the ENPs.

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Figure captions

Fig. 1 Concentration of available mineral nutrients in soils without (dark blue color) and with plants (light blue color): a) Mn soil porewater; b) Mn available; c) Fe available; d) P available. Bars represent mean values ± sd (n=4); for each group different letters are significantly different at $p<0.05$ according to two way ANOVA and post hoc Tukey test. Asterisks denote that significantly differences occurred between C1 and C2 at $p< 0.05$ according to T-student test. C1=control soil; C2=amended soil control; A, R, Mix, B=amended soil spiked with anatase, rutile, mixture of A+R (1:1 ratio) and bulk, respectively; 80, 800=low, high dose TiO_2 treatments.

Fig. 2 a) Length and b) Dry biomass of the roots (brown color) and the shoots (green color) of pea plants. Bars represent mean values ± sd (n=4); for each group different letters are significantly different at $p<0.05$ according to two way ANOVA and post hoc Tukey test. Asterisks denote that significantly differences occurred between the controls (C1 and C2) at $p< 0.05$ according to T-student test. C1=control soil; C2=amended soil control; A, R, Mix, B=amended soil spiked with anatase, rutile, mixture of A+R (1:1 ratio) and bulk, respectively; 80, 800=low, high dose TiO_2 treatments. F and p values from Anova (α=0.05) for dose, treatment and interaction of variables. Boldface indicates statistically significant differences.

Fig. 3 Concentration of mineral nutrients in the roots (brown color) and the shoots (green color) of pea plants: a) Mn; b) K; c) Zn; d) P. Bars represent mean values ± sd (n=4); for each group different letters are significantly different at $p<0.05$ according to two way ANOVA and post hoc Tukey test. Asterisks denote significant differences between controls (C1 and C2) at $p<0.05$, according to T-student test. C1=control soil; C2=amended soil control; A, R, Mix, B=amended soil spiked with anatase, rutile, mixture of A+R (1:1 ratio) and bulk, respectively; 80, 800=low, high dose TiO_2 treatments.

Fig. 4 Pigments as total chlorophyll (green color) and carotenoids (orange color) in leaves of pea plants. Bars represent mean values ± sd (n=4); for each group different letters are significantly different at $p<0.05$ according to two way ANOVA and post hoc Tukey test. Asterisks denote that significant differences occurred between the controls (C1 and C2) at $p<0.05$, according to T-student test. C1=control soil; C2=amended soil control; A, R, Mix, B=amended soil spiked with anatase, rutile, mixture of A+R (1:1 ratio) and bulk, respectively; 80, 800=low, high dose TiO_2 treatments. F and p values from Anova (α=0.05) for dose, treatment and interaction of variables. Boldface indicates statistically significant differences.

Table 1

Table 1

Main properties (dw basis) of the control soil (C1), the biosolid (Bs) and the amended soil control (C2). Total concentration of mineral nutrients are mean values with standard deviation (n=4).

parameter	**C1**	**Bs**	**C2**
solid residue at 105°C (%)	99.0	18.0	98.8
pH (H_2O)	7.70	6.92	7.01
sand (%)	93.3	-	93.4
silt (%)	4.6	-	4.5
clay (%)	2.1	-	2.1
EC (mS cm^{-1})	0.80	11.5	2.1
CEC ($cmol^{(+)}$ kg^{-1})	15.4	-	23.4
OM (%)	1.1	57.3	3.4
C_{org} (%)	0.61	33.3	2.0
C_{inorg} (%)	0.16	-	0.40
N_{tot} (mg kg^{-1})	404 ± 3.28	49003 ± 803	2303 ± 6.11
P_{tot} (mg kg^{-1})	263 ± 5.36	20030 ± 770	890 ± 18.5
K_{tot} (mg kg^{-1})	7350 ± 150	4632 ± 238	7460 ± 197
Ca (mg kg^{-1})	3300 ± 70.8	21653 ± 461	3950 ± 128
Mg (mg kg^{-1})	1557 ± 113	12400 ± 395	3508 ± 205
Fe (mg kg^{-1})	10393 ± 89.7	11486 ± 391	10739 ± 197
Mn (mg kg^{-1})	330 ± 4.35	106 ± 10.2	337 ± 7.45
Cu (mg kg^{-1})	6.55 ± 0.11	248 ± 11.4	48.0 ± 2.56
Zn (mg kg^{-1})	32.9 ± 2.42	627 ± 12.5	137 ± 9.67

EC=electrical conductivity; CEC=cation exchange capacity; OM=organic matter

Table 2
Titanium concentration in the different soils and in the Bs, represented as mean values with standard deviation (n=4). Different letters are significantly different at $p<0.05$ according to ANOVA and post hoc Tukey test.

matrix	**Ti content (mg kg^{-1} dw)**
Bs	699 ± 105 a
C1	1529 ± 152 b
C2	1657 ± 127 b
A80	1605 ± 145 b
A800	2190 ± 112 c
R80	1586 ± 106 b
R800	2040 ± 186 c
Mix80	1468 ± 116 b
Mix800	2201 ± 95 c
B80	1517 ± 59 b
B800	2072 ± 110 c

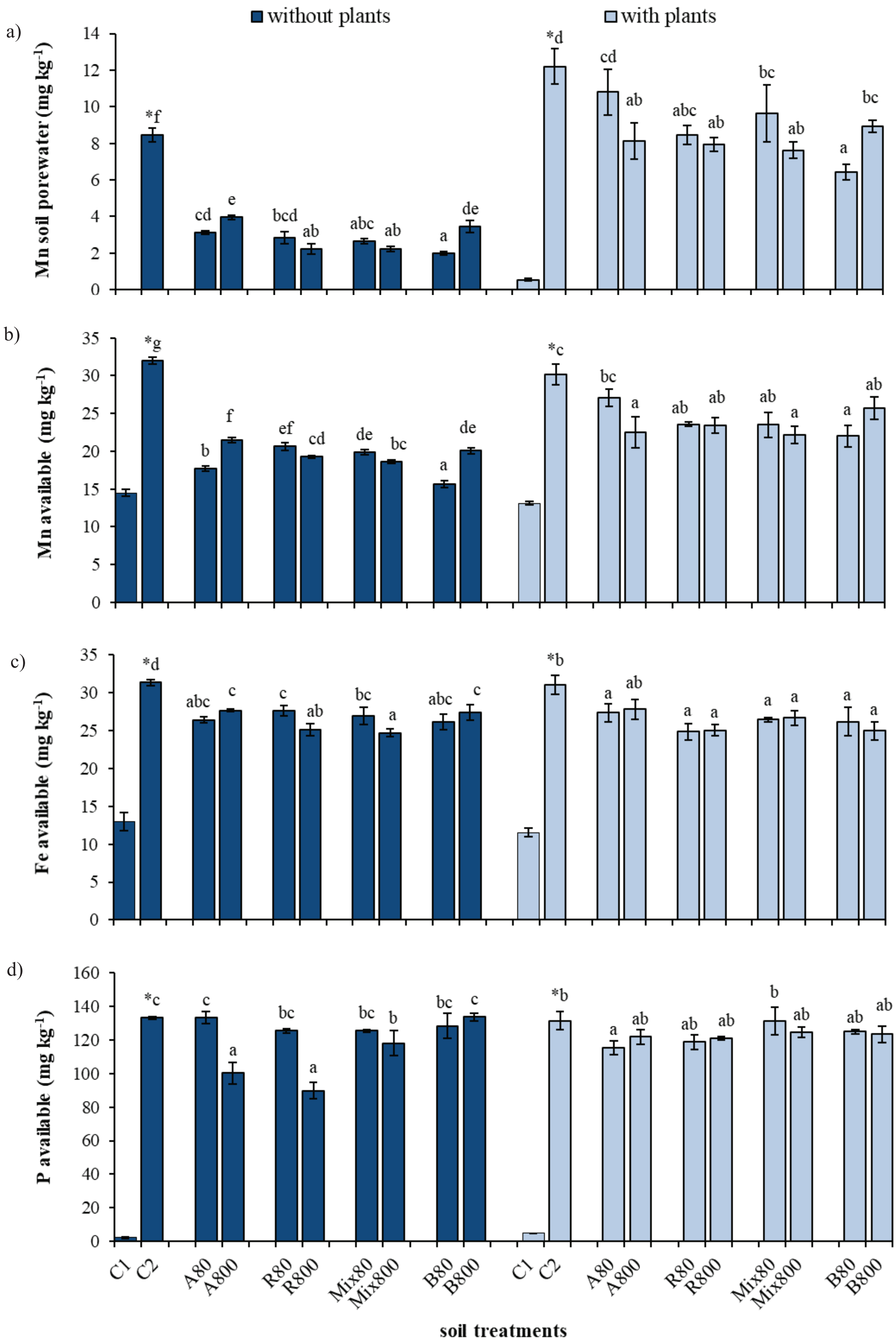
Bs= biosolid; C1=control soil; C2=amended soil control; A, R, Mix, B= amended soil spiked with anatase, rutile, mixture of A+R (1:1 ratio) and bulk, respectively; 80, 800=low, high dose TiO_2 treatments.

Table 3
Shannon Weaver (H) and Evenness (E) Indexes of the bacterial community in a) low and b) high dose treatments. Values followed by different letter in the same column and for the same treatment are significantly different at 5% level by using the Bonferroni correction. Asterisk denotes significant difference between C1 and C2 according to Student t-test.

	soil treatments		Shannon Weaver Index (H)	Evenness Index (E)
a)	controls	C1	4,27	0,81
		C2	4,35* c	0,81 a
	without plants	A80	4,46 d	0,90 c
		R80	6,44 i	0,92 d
		Mix80	0,47 a	0,89 b
		B80	4,90 f	0,93 e
	with plants	A80	4,64 e	0,92 d
		R80	5,01 g	0,92 d
		Mix80	5,58 h	0,93 e
		B80	4,08 b	0,95 f a
b)	controls	C1	3,20	0,93
		C2	3,28* g	0,93 b
	without plants	A800	3,29 h	0,93 b
		R800	3,25 f	0,94 c
		Mix800	3,12 b	0,99 f
		B800	3,29 h	0,93 b
	with plants	A800	3,16 d	0,97 e
		R800	3,21 e	0,96 d
		Mix800	2,92 a	0,71 a
		B800	3,13 c	0,99 f

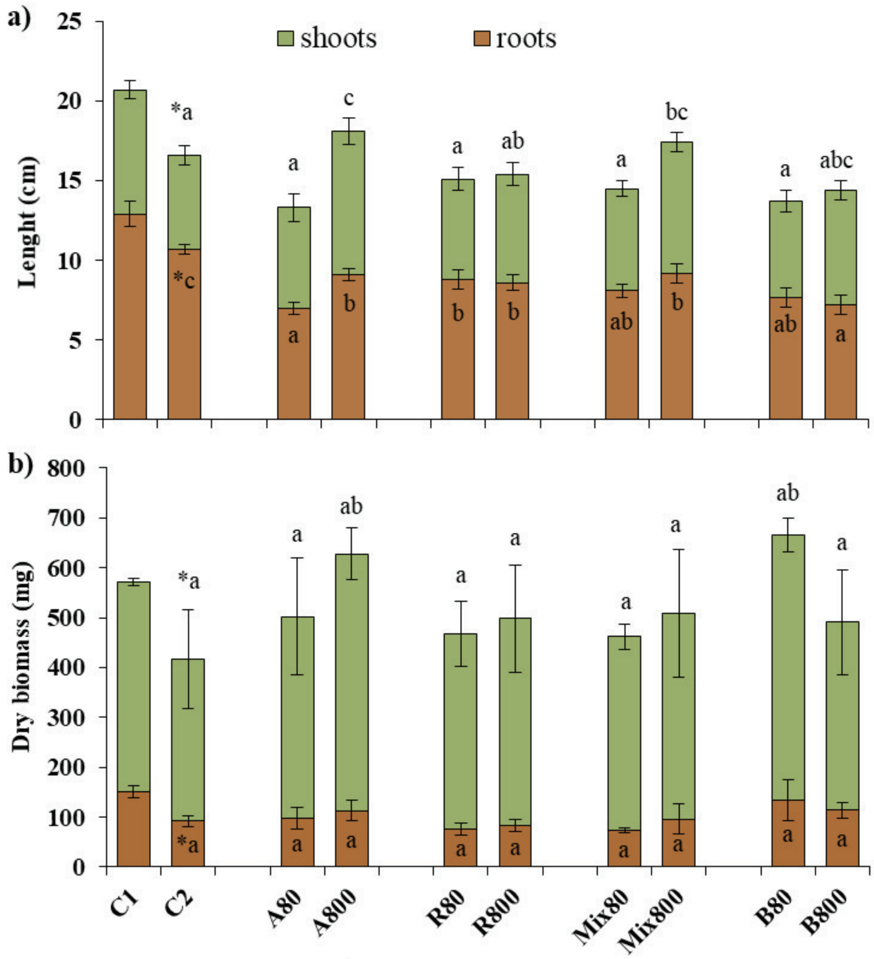
C1=control soil; C2=amended soil control; A, R, Mix, B=amended soil spiked with anatase, rutile, mixture of A+R (1:1 ratio) and bulk, respectively; 80, 800=low, high dose TiO_2 treatments.

Figure 1



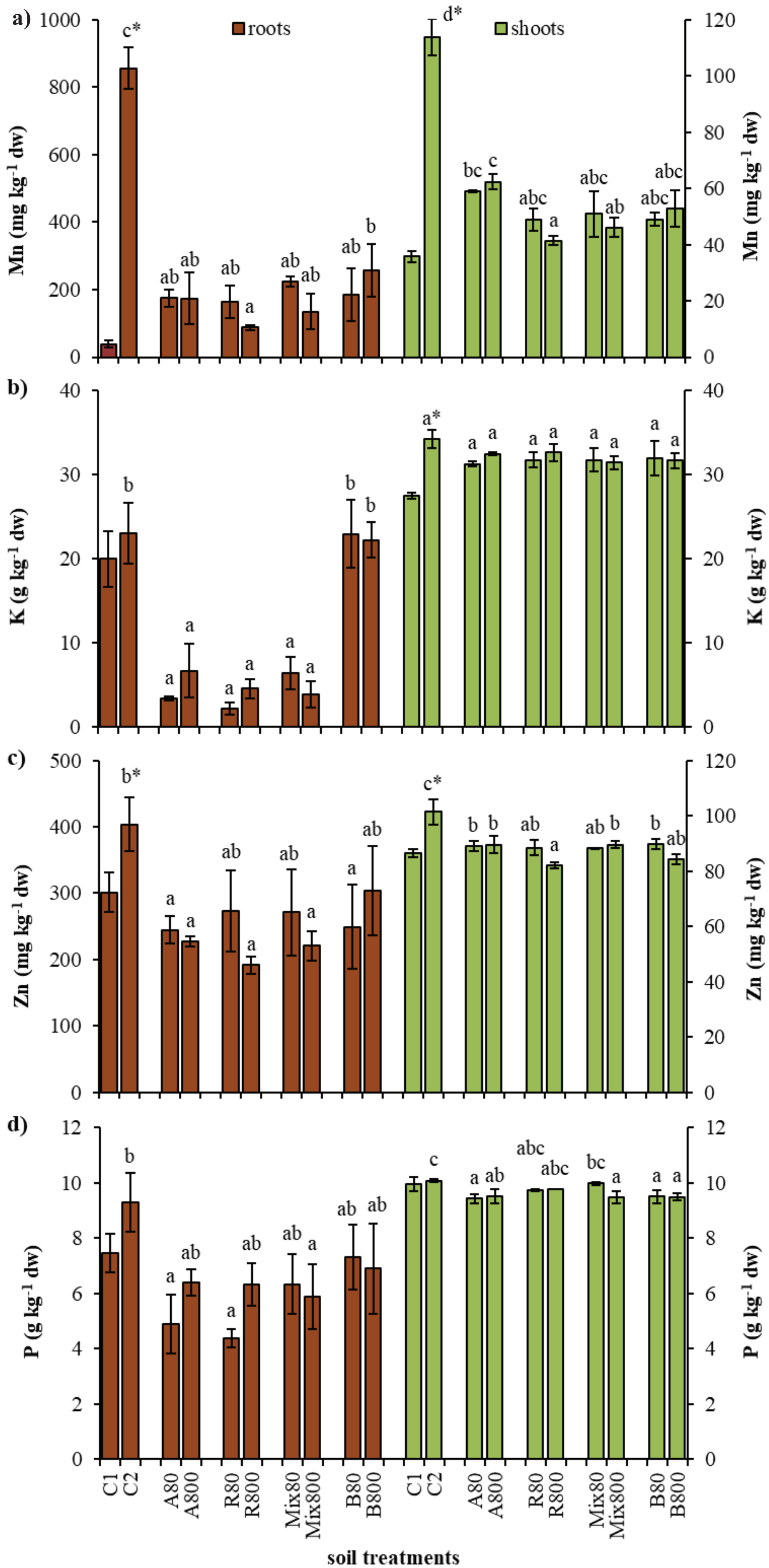
without plants
with plants
a)
Mn soil porewater (mg kg-1)
14
12
10
8
6
4
2
0
*f
cd
e
bcd
ab
abc
ab
a
de
*d
cd
ab
abc
ab
bc
ab
a
bc
b)
Mn available (mg kg-1)
35
30
25
20
15
10
5
0
*g
b
f
ef
cd
de
bc
a
de
*c
bc
a
ab
ab
ab
a
a
ab
c)
Fe available (mg kg-1)
35
30
25
20
15
10
5
0
*d
abc
c
c
ab
bc
a
abc
c
*b
a
ab
a
a
a
a
a
a
d)
P available (mg kg-1)
160
140
120
100
80
60
40
20
0
*c
c
a
bc
a
bc
b
bc
c
*b
a
ab
ab
ab
b
ab
ab
ab
C1
C2
A80
A800
R80
R800
Mix80
Mix800
B80
B800
C1
C2
A80
A800
R80
R800
Mix80
Mix800
B80
B800
soil treatments

Figure 2



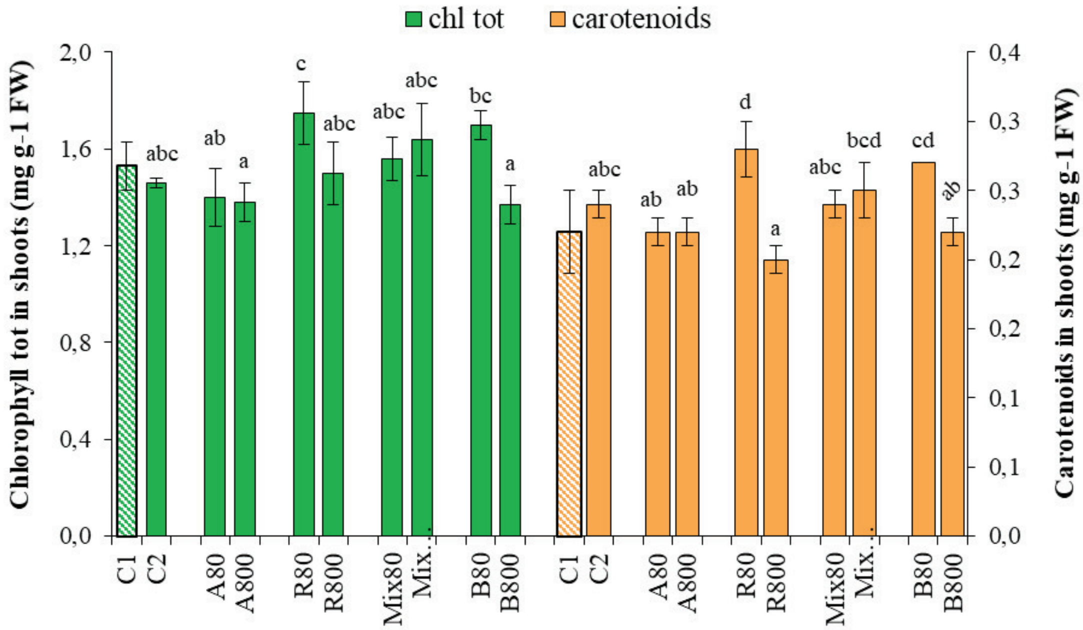
		Roots		Shoots	
		F value	*p value*	*F value*	*p value*
lenght	dose	**18.1**	**0.003**	**13.0**	**0.007**
	treatments	0.97	0.47	1.23	0.38
dry biomass	dose	0.39	0.69	4.31	0.07
	treatments	2.40	0.16	0.40	0.76

Figure 3



a)
roots
shoots
Mn (mg kg-1 dw)
b)
K (g kg-1 dw)
c)
Zn (mg kg-1 dw)
d)
P (g kg-1 dw)
C1
C2
A80
A800
R80
R800
Mix80
Mix800
B80
B800
soil treatments

Figure 4



	Chlorophyll tot			Carotenoids		
	dose	treatments	interaction	dose	treatments	interaction
F value	2,96	1,80	1,57	**5,85**	0,68	2,21
p value	0,07	0,17	0,20	**0,009**	0,57	0,08

Supplementary Material 1
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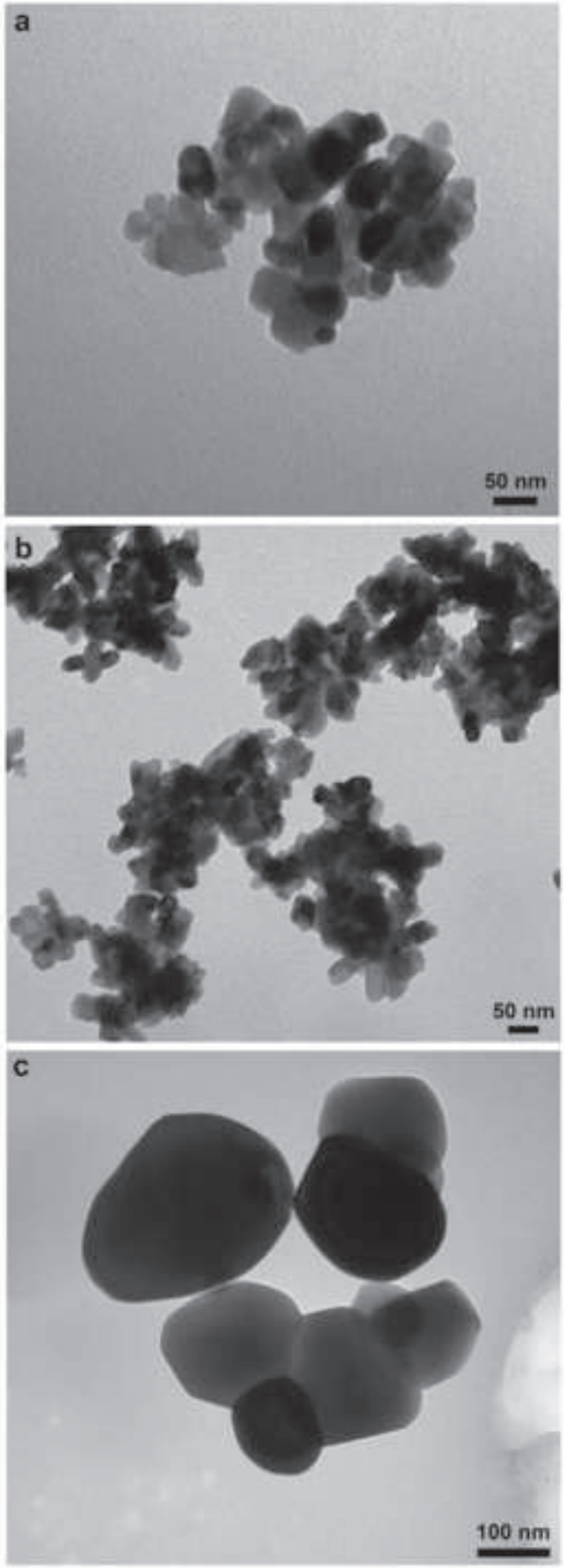

Supplementary Material 2

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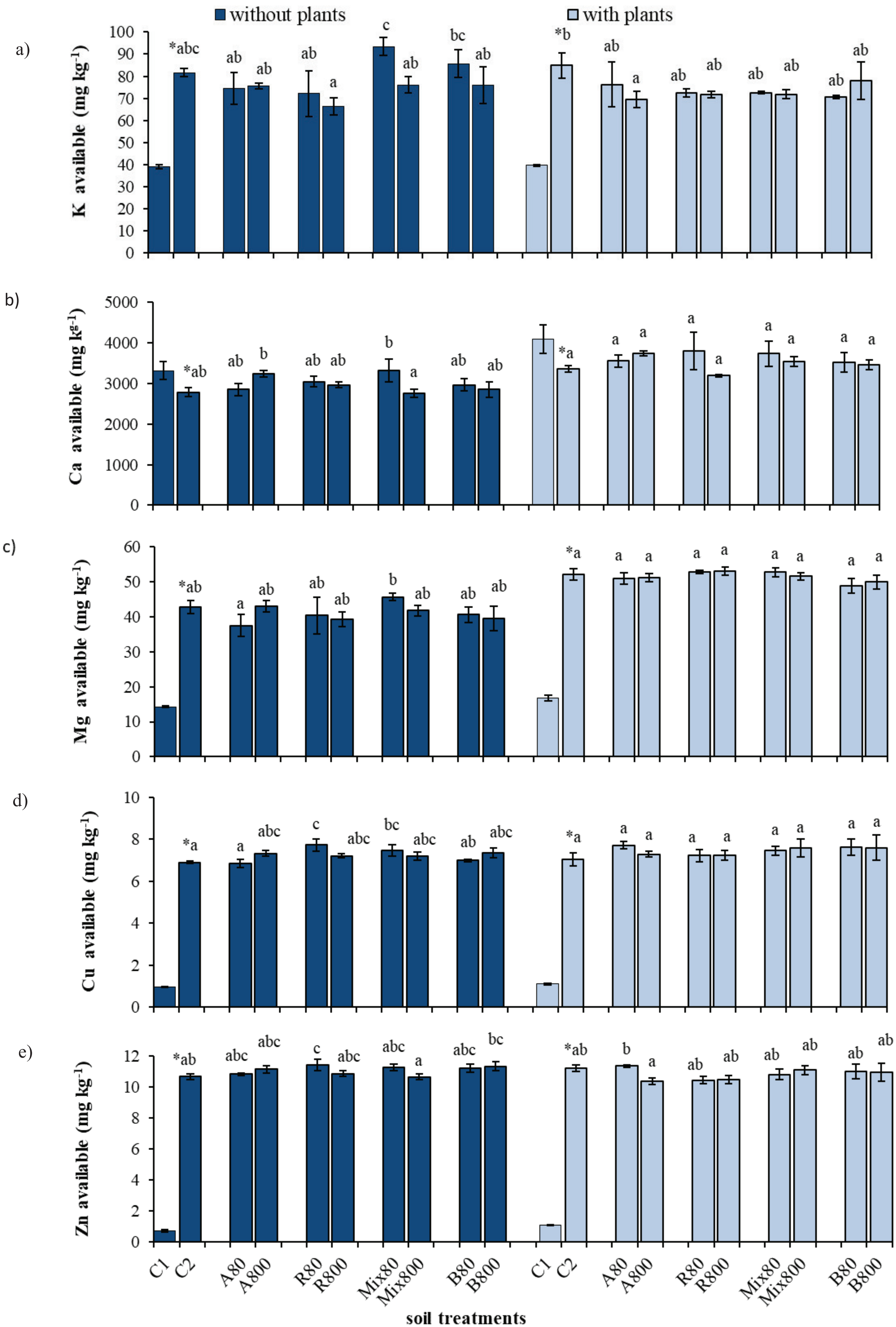

Supplementary Material 3

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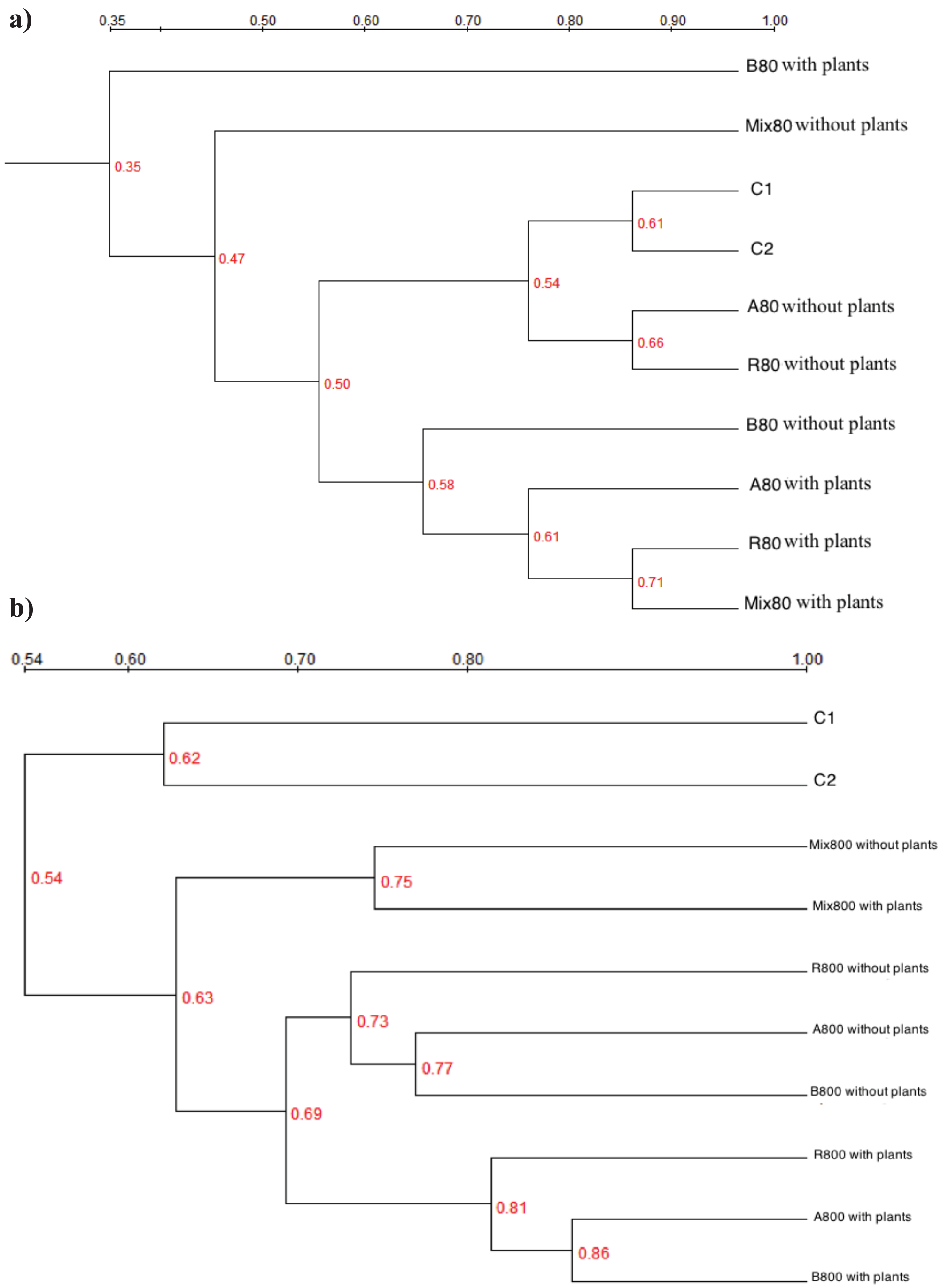

Supplementary Material 4

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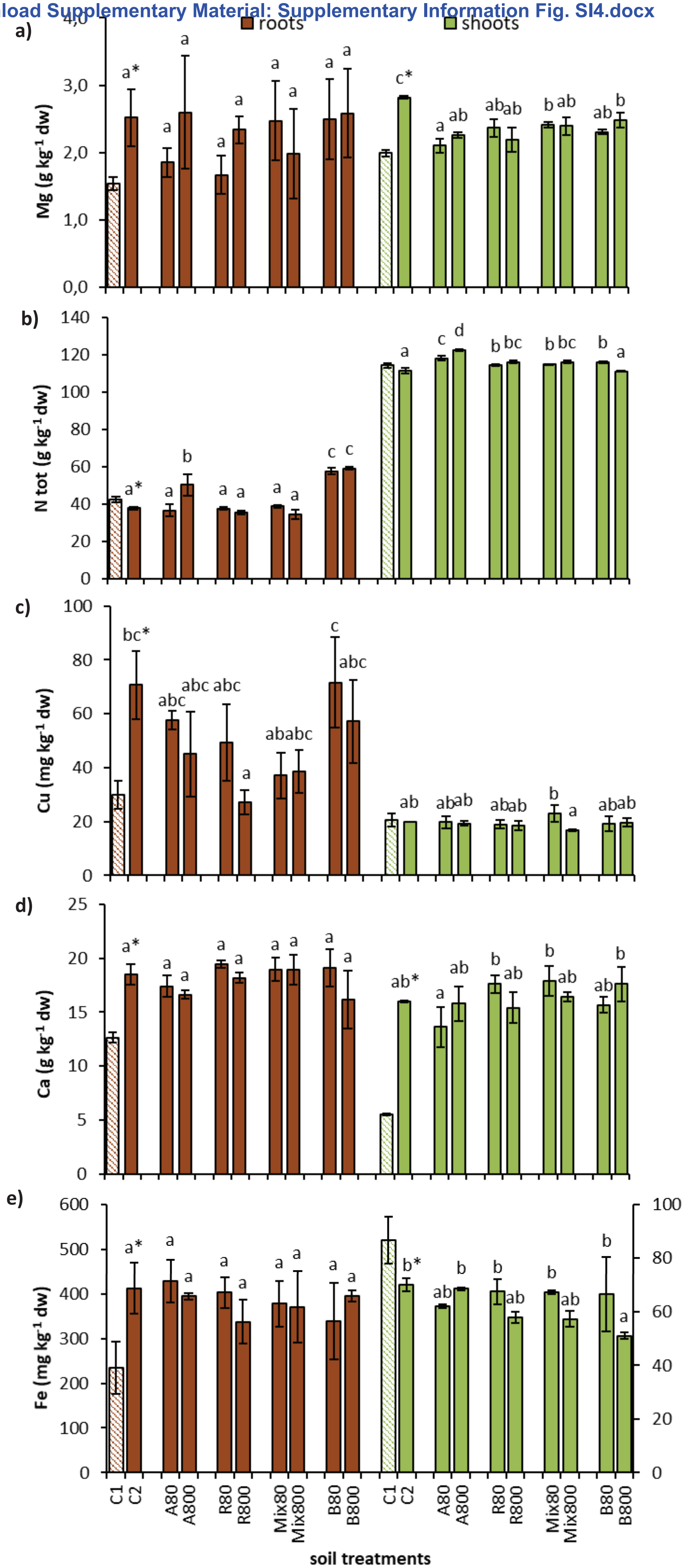

Supplementary Material 5
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Supplementary Information - Fig. SI 1 TEM images of pristine TiO_2 aggregates: a) anatase nanoparticles; b) rutile nanoparticles; c) bulk particles.

Supplementary Information - Fig. SI 2 Concentration of available mineral nutrients in soils without (dark blue color) and with plants (light blue color): a) $K_{available}$; b) $Ca_{available}$; c) $Mg_{available}$; d) $Cu_{available}$; e) $Zn_{available}$. Bars represent mean values ± sd (n=4); for each group different letters are significantly different at $p<0.05$ according to two way ANOVA and post hoc Tukey test. Asterisks denote that significantly differences occurred between C1 and C2 at $p< 0.05$ according to T-student test. C1=control soil; C2=amended soil control; A, R, Mix, B=amended soil spiked with anatase, rutile, mixture of A+R (1:1 ratio) and bulk, respectively; 80, 800=low, high dose TiO_2 treatments.

Supplementary Information - Fig. SI 3 Dendrogram plots of 16S rDNA PCR-DGGE of the bacterial community in soils without and with plants at: a) low and b) high dose treatments. C1=control soil; C2=amended soil control; A, R, Mix, B=amended soil spiked with anatase, rutile, mixture of A+R (1:1 ratio) and bulk, respectively; 80, 800=low, high dose TiO_2 treatments.

Supplementary Information - Fig. SI 4 Concentration of mineral nutrients in the roots (brown color) and the shoots (green color) of pea plants: a) Mg; b) N_{tot}; c) Cu; d) Ca; e) Fe. Bars represent mean values ± sd (n=4); for each group different letters are significantly different at $p<0.05$ according to two way ANOVA and post hoc Tukey test. Asterisks denote significantly differences between controls (C1 and C2) at $p<0.05$, according to T-student test. C1=control soil; C2=amended soil control; A, R, Mix, B=amended soil spiked with anatase, rutile, mixture of A+R (1:1 ratio) and bulk, respectively; 80, 800=low, high dose TiO_2 treatments.

Supplementary Material 6

Click here to download Supplementary Material: Supplementary Information Table SI1.doc

Supplementary information - Table SI1

F and *p* values from Anova (α=0.05) for the low and high Ti doses (80 and 800 mg kg^{-1} TiO_2, respectively), the treatments and the interaction of variables in (a) available soil nutrients with (+) and without (-) plants, and (b) nutrients in roots and shoots. Boldface indicates statistically significant differences.

a) soil nutrients		*F value*	*p value*
Mn -plants (porewater)	Ti dose	**1574**	**$3.5\ 10^{-26}$**
	Treatment	**12.3**	**$4.5\ 10^{-5}$**
	Interaction	**12.3**	**$2.7\ 10^{-6}$**
Mn +plants (porewater)	Ti dose	**71.0**	**$8.3\ 10^{-11}$**
	Treatment	2.89	0.06
	Interaction	**5.74**	**0.0008**
Mn -plants (potentialy avail.)	Ti dose	**4539**	**$1.1\ 10^{-31}$**
	Treatment	**23.1**	**$2.9\ 10^{-7}$**
	Interaction	**56.7**	**$5.4\ 10^{-13}$**
Mn +plants (potentialy avail.)	Ti dose	**89.1**	**$7.8\ 10^{-12}$**
	Treatment	1.44	0.255
	Interaction	**5.13**	**0.002**
Fe -plants	Ti dose	**201**	**$9.9\ 10^{-16}$**
	Treatment	2.36	0.097
	Interaction	**7.39**	**0.0001**
Fe +plants	Ti dose	**67.5**	**$1.4\ 10^{-10}$**
	Treatment	**3.85**	**0.022**
	Interaction	1.28	0.303
P -plants	Ti dose	**105**	**$1.39\ 10^{-12}$**
	Treatment	**23.5**	**$2.59\ 10^{-7}$**
	Interaction	**24.2**	**$4.6\ 10^{-9}$**
P +plants	Ti dose	**13.7**	**0.0001**
	Treatment	**3.14**	**0.044**
	Interaction	1.82	0.138
K -plants	Ti dose	**9.43**	**0.001**
	Treatment	**6.65**	**0.002**
	Interaction	**3.30**	**0.016**
K +plants	Ti dose	**20.9**	**$5.6\ 10^{-6}$**
	Treatment	0.139	0.94
	Interaction	0.990	0.46
Ca -plants	Ti dose	**6.61**	**0.005**
	Treatment	0.49	0.69
	Interaction	**3.65**	**0.01**
Ca +plants	Ti dose	**6.75**	**0.005**
	Treatment	0.77	0.52
	Interaction	2.35	0.06
Mg -plants	Ti dose	2.02	0.15
	Treatment	2.06	0.13
	Interaction	2.31	0.07
Mg +plants	Ti dose	0.88	0.43
	Treatment	**3.95**	**0.02**
	Interaction	1.25	0.32
Cu -plants	Ti dose	**18.7**	**$1.29\ 10^{-5}$**
	Treatment	**4.05**	**0.018**
	Interaction	**7.17**	**0.0002**
Cu +plants	Ti dose	**7.00**	**0.004**
	Treatment	1.08	0.38
	Interaction	0.69	0.66
Zn -plants	Ti dose	**15.6**	**0.00005**
	Treatment	1.82	0.17
	Interaction	**3.68**	**0.010**
Zn +plants	Ti dose	**7.26**	**0.003**
	Treatment	2.57	0.078
	Interaction	**3.01**	**0.024**

b) plant nutrients		*F value*	*p value*
Mn roots	Ti dose	**560**	**$7.2\ 10^{-21}$**
	Treatment	1.87	0.16
	Interaction	1.74	0.16
Mn shoots	Ti dose	**427**	**$1.7\ 10^{-19}$**
	Treatment	**4.85**	**0.009**
	Interaction	1.89	0.12
K roots	Ti dose	**119**	**$3.6\ 10^{-13}$**
	Treatment	**47.1**	**$3.3\ 10^{-10}$**
	Interaction	**12.6**	**$2.2\ 10^{-6}$**
K shoots	Ti dose	**19.6**	**$8.9\ 10^{-6}$**
	Treatment	0.21	0.88
	Interaction	0.45	0.84
Zn roots	Ti dose	**48.7**	**$3.6\ 10^{-9}$**
	Treatment	0.78	0.51
	Interaction	1.44	0.24
Zn shoots	Ti dose	**77.6**	**$3.3\ 10^{-11}$**
	Treatment	1.39	0.27
	Interaction	1.58	0.20
P roots	Ti dose	**39.6**	**$2.5\ 10^{-8}$**
	Treatment	2.15	0.12
	Interaction	1.62	0.18
P shoots	Ti dose	**43.5**	**$1.0\ 10^{-8}$**
	Treatment	**4.21**	**0.016**
	Interaction	**3.75**	**0.009**
Mg roots	Ti dose	0.59	0.56
	Treatment	1.04	0.39
	Interaction	0.88	0.53
Mg shoots	Ti dose	**130**	**$1.3\ 10^{-13}$**
	Treatment	**5.40**	**0.006**
	Interaction	**3.93**	**0.007**
N roots	Ti dose	**32.7**	**$1.4\ 10^{-7}$**
	Treatment	**87.7**	**$4.5\ 10^{-13}$**
	Interaction	**32.1**	**$2.5\ 10^{-10}$**
N shoots	Ti dose	**90.6**	**$6.6\ 10^{-12}$**
	Treatment	**35.9**	**$4.9\ 10^{-9}$**
	Interaction	**21.2**	**$1.7\ 10^{-8}$**
Cu roots	Ti dose	**16.9**	**$2.62\ 10^{-5}$**
	Treatment	**4.29**	**0.015**
	Interaction	1.56	0.20
Cu shoots	Ti dose	3.38	0.051
	Treatment	0.33	0.80
	Interaction	2.89	0.029
Ca roots	Ti dose	**3.42**	**0.049**
	Treatment	2.27	0.11
	Interaction	1.33	0.28
Ca shoots	Ti dose	0.29	0.75
	Treatment	**3.88**	**0.022**
	Interaction	**4.23**	**0.005**
Fe roots	Ti dose	1.53	0.23
	Treatment	0.58	0.63
	Interaction	0.83	0.56
Fe shoots	Ti dose	**18.8**	**$1.24\ 10^{-5}$**
	Treatment	1.37	0.28
	Interaction	**3.58**	**0.011**